Title: METHOD FOR THE PREPARATION AND USE OF AN ENRICHED CYANIDINE-5-O-BETA-GLUCOPYRANOSIDE EXTRACT AND DERIVATIVES THEREOF FROM FRUITS AND VEGETABLES CONTAINING SAID ANTHOCYANIN AND METHOD FOR THE PURIFICATION AND USE OF CYANIDINE-3-O-BETA-GLUCOPYRANOSIDE AND DERIVATIVES THEREOF OBTAINED FROM SAID EXTRACT

Abstract: The present invention concerns a method for the preparation and the use of an extract enriched of cyanidin-3-O-beta-glucopyranoside and of its derivatives obtained from fruits or vegetables containing the aforementioned anthocyanin, and for the purification and the use of cyanidin-3-O-beta-glucopyranoside and of its derivatives from this extract. The extract enriched of cyanidin-3-O-beta-glucopyranosideand of its malonylated cyanidin-3-O-beta-glucopyranoside derivative, free of any toxic compound, can be used as a dietary supplement (nutraceutical), whilst the cyanidin-3-O-beta-glucopyranoside derivative can be obtained at a purity degree suitable for the preparation of pharmaceutical products.
METHOD FOR THE PREPARATION AND USE OF AN ENRICHED CYANIDINE-3-O-BETA-GLUCOPYRANOSIDE EXTRACT AND DERIVATIVES THEREOF FROM FRUITS AND VEGETABLES CONTAINING SAID ANTHOCYANIN AND METHOD FOR THE PURIFICATION AND USE OF CYANIDINE-3-O-BETA-GLUCOPYRANOSIDE AND DERIVATIVES THEREOF OBTAINED FROM SAID EXTRACT

The present invention concerns a method for the preparation and use of an extract enriched in cyanidin-3-O-β-glucopyranoside and of its derivatives from fruits and vegetables containing this anthocyanin, and for the purification and use of cyanidin-3-O-β-glucopyranoside and of its derivatives from this extract. In particular, the method according to the invention allows the preparation of an extract enriched in cyanidin-3-O-β-glucopyranoside and of its malonylated cyanidin-3-O-β-glucopyranoside derivative, free of any toxic compound, that can be advantageously used as a dietary supplement (nutraceutical) and the purification of cyanidin-3-O-β-glucopyranoside and its malonylated cyanidin-3-O-β-glucopyranoside derivative having such a purity degree to permit the preparation of pharmaceutical products.

Anthocyanins are a family of pigments in which a molecule of an anthocyan is linked to a molecule of a carbohydrate; the saccharidic residue renders these compounds highly water-soluble. Anthocyanins are responsible for the color of several fruits and flowers; in fact, the red color nuance with tendency to purple are due to anthocyanes (these are present in apples, cherries, peaches, grape, red radicchio, radishes), whilst the red color nuancing towards orange (such as in tomatoes, peppers, etc.) is mainly due to the presence of carotenoids localized within the plastids. In the plant kingdom, the main biological role of anthocyanins is to attract animal for the pollination process and the subsequent seed dispersion. As previously mentioned, anthocyanins are diffused in various amounts also in different fruits; in particular, they are responsible for the color appearing in the red orange from Sicily, which represent about the 70% of the whole Italian orange production.

From a structural point of view, anthocyanins are characterized by the basic cationic structure of the flavylum salt, with different substitutions on the B ring. The electron deficiency of their structure, renders anthocyanins highly reactive and their stability is pH and
temperature-dependent (1). The saccaridic residue of anthocyanins can be linked to the carbon in 3, 5, 7, 3’ 5’, and the most common monosaccaride are glucose, ramnose, galactose, xylulose and arabinose. The anthocyanin free of its saccaridic moiety is named aglycone. It should be underlined that, due to the simultaneous presence in the same molecule of numerous OH- groups, anthocyanins are also considered as polyphenols from a chemical point of view. Among the different anthocyanins, our interest is focused on cyanidin-3-O-β-glucopyranoside (C-3-G) which is an anthocyanin present in various fruits and vegetables, as for example strawberry, blueberry, blackberry, cherry, rhubarb, red turnip, red onion, black mulberries etc. (2, 3), as well as in red oranges (of the cultivar named Moro, Sanguinello and Tarocco) (4) mainly growing in Sicily and, in a small amount, in the Malta island too. In the aforementioned cultivar of the red orange, C-3-G represents about the 90% of the total anthocyanin content, reaching a remarkable relative abundance (up to 500-600 mg/l of juice). To this purpose, it is worth mentioning that the red orange is a type of fruit still few considered that about the 50% of the total harvest is destroyed, in spite of its chemical composition that renders this orange cultivars absolutely peculiar from a nutritional point of view.

As below indicated, the structure of C-3-G has two OH-groups in the positions 3’- and 4’- of the B ring (for a total of 4 hydroxyl groups per each C-3-G molecule) and a β-D-glucose residue in the pyranosic form linked to the C ring by means of a 3-O-β glycosidic bond.
In the last years, great attention has been given to the potential protection exerted by natural antioxidants, which are contained in the vegetable diet, towards cellular and tissue damages induced by the action of reactive oxygen species (ROS) (5, 6), with particular consideration to the effects of flavonoids and polyphenols (7-9). In addition, even if some concerns have been raised because of their instability at physiological pH, data obtained in recent experimental research let to consider anthocyanins as natural substances with protective effects of great relevance (1, 10).

In fact, it has been suggested that anthocyanins have a remarkable activity as scavengers of oxygen free radicals, even much higher than that of other natural antioxidants. The antioxidant potency of anthocyanins should be dependent on their chemical structure, even if the mechanism of action and the structure-function relationship of the different anthocyanins have not yet been fully clarified. In fact, it has been reported that, by varying either the position or the type of chemical groups linked to the aromatic rings of anthocyanins, it is possible to modulate their capacity to accept unpaired electrons from radical molecules and, consequently, to increase or decrease the corresponding antioxidant power (1, 10). The O-diphenolic substitution on the B ring of the anthocyanins and the conjugated double bonds system are correlated to their scavenging radical activity caused by the loss of the H⁺ and the subsequent radical stabilization. In other cases, the presence of additional hydroxyl groups does not increase the antioxidant properties of anthocyanins, as evaluated in vitro on human LDL peroxidation. These data are of difficult interpretation and have not yet allowed to find a definitive correlation between structure and function of anthocyanins (1, 11). Among the various anthocyanins, C-3-G has been indicated as the one having the major antioxidant capacity (1, 10, 12-15); its mechanism of action, at least as far as the metal-dependent systems for generating reactive oxygen species (ROS), has been mainly attributed to its capacity to chelate divalent metal ions necessary to produce ROS through the Fenton reaction (1). This mechanism postulates that the C-3-G generates the complex with metals through the ionization of the hydroxyl groups of the B ring, however, this phenomenon at pH 7.4 might take place only in minimal level because of weak acidic character of phenols. Recently, it has been obtained a better characterization of both the antioxidant profile and the mechanism of action of C-3-G, as for example its interaction with hydroxyl radicals,
superoxide anions and hydrogen peroxide, and the value of its redox potential (16). Further studies, always carried out by our research group, evidenced the ability of C-3-G to decrease the damages induced by ischemia and reperfusion on isolated rat heart and the peroxidative damage induced by hydrogen peroxide in isolated human erythrocytes (17, 18). Furthermore, we also evidenced a new C-3-G effect correlated to its capacity to differentiate human melanoma cells; in these experiments, C-3-G has been able to produce this differentiating effect through the activation of specific genes responsible for the cell differentiation. This result might certainly open new potential pharmacological and nutritional application of C-3-G (19).

Up to now, it is not available a process which, starting from low cost sources, as for example the juice of blood oranges of Sicily (or other similar natural sources having a higher cost and a higher yield as black mulberries, the water melon juice, the red cherries juice, etc.), allows to obtain C-3-G in such a purity degree usable to produce C-3-G-based drugs.

At present, the existing procedures do not permit to obtain, at an intermediate stage of the purification process, a lyophilized preparation containing C-3-G and vitamin C in such a concentrations that this preparation is used as a dietary supplement (nutraceutical).

The only lyophilized preparation obtained from the juice of blood oranges containing C-3-G, needs more complex methods and does not allow to obtain a final product free of any contamination with compounds potentially damaging for human health.

C-3-G-containing extracts obtained from sources other than juice of blood oranges (such as bilberry) and nowadays on market, are composed by different types of anthocyanins each of them having antioxidant activity which varies from anthocyanin to anthocyanin.

In light of what aforementioned it is therefore evident the necessity of having a method of extraction of cyanidin-3-O-β-glucopyranoside and of its derivatives overcoming the disadvantages of the known techniques.

On the basis of the scientific premises related to the biological properties of C-3-G, the Authors of the present invention set up (at the laboratory level) a simple method, characterized by low cost and high yield, for the extraction and purification of C-3-G from blood orange juice
of Sicily, method that can be applied to any other extract of natural origin containing significant amount (significant from an industrial point of view) of C-3-G and of its malonylated derivative. The method object of the present invention can be also applied to obtained a final product containing C-3-G at a very high purity degree (about the 99%) that is suitable to prepare pharmaceutical drugs formed of C-3-G, or this method can be applied as well to obtained a lyophilized product that can be used as a dietary supplement formed by C-3-G, malonylated C-3-G and vitamin C (ascorbic acid).

The juice of blood oranges of Sicily represents the best choice to extract and purify C-3-G either because this molecule represents about the 90% of the total anthocyanine juice content, either because the cost and the abundance of blood orange juice is much lower and much higher, respectively, than the cost and the abundance of other possible natural sources.

The method object of the present invention allows to decide the level of extraction and purification of the C-3-G on the basis of the productive needs linked to the market request and, for the first time, it allows to produce a product to be used as a dietary supplement (nutraceutical) composed by the simultaneous presence of C-3-G, its malonylated derivative and ascorbic acid that play a synergistic effect which reciprocally increases the respective antioxidant capacities.

In addition, the invention concerns a method of industrial production totally free of manipulation of the blood orange juice with organic solvents, at least up to the level of preparation of the lyophilized product usable as a dietary supplement. The further purification steps to have a C-3-G-based product usable for pharmaceutical drugs require the use of organic solvents easily and fully removable (methanol and formic acid), both solvents largely in use in the pharmaceutical industry, either in the synthesis processes or in the purification procedures.

In addition, the method object of the present invention guarantees to obtain a product to be used as a dietary supplement composed by a single anthocyanin, the cyanidin, in its glycosylated forms (C-3-G) and concomitantly glycosylated and malonylated (malonylated C-3-G), whilst at the highest purity achievable it is possible to obtain monocomponent products made of C-3-G only or malonylated C-3-G only (the choice to mix C-3-G with malonylated C-3-G, logically permitted because
of the equal antioxidant potency of the two compounds, strictly depends on industrial/bureaucratic choices).

It is therefore the specific object of the present invention a method for the preparation of an extract enriched of cyanidin-3-O-β-glucopyranoside and of its derivatives obtained from fruits such as blood oranges, strawberries, bilberries, blackberries, cherries, black mulberry, raspberry, water melon, back grape, strawberry grape, or from vegetables such as rhubarb, red turnip, red onions, red rice containing this compound, the method comprising the following steps:

1. withdrawal of juice from fruits or vegetables, for example by squeezing, and removal of the possible presence of particulate matter, for example by centrifugation or simple filtration through a filtering device made of inert material such as porous glass, filtering gauzes, filtering paper;

2. filtration of the liquid obtained by step 1) by using reversed osmosis or by means of ultrafiltration/nanofiltration, using a membrane, for example a polymeric or ceramic-made membrane, with a molecular cut-off of filtration for uncharged solutes of 100-800 Da, preferably 200-500 Da, even more preferably 300 Da; and

3. collection of filtrate.

The method according the invention can further comprise the step 4) of lyophilization of the filtrate or of any other alternative technique suitable for water removal, including the “spray-drying” technique. In addition, the method can comprise a further step 5) of purification of C-3-G, subsequent to step 3) or alternative to it, that can be effected in continuous at the end of the filtration step 2).

As far as the filtration step 2) is concerned, the polymer of the polymeric membrane for the reversed osmosis is selected in the group consisting of cellulose acetate, polyamide, polyethersulfone, whilst the polymeric membrane for reversed osmosis is chosen in the group of a tangential flow spiral-wrapped membrane, hollow-fiber membrane. Membranes for ultrafiltration/nanofiltration are represented by ceramic membranes. The filtration can be carried out at a pressure ranging from 5 to 40 bar, preferably from 15 to 35 bar, even more preferably at 27 bar, and with flow permeability ranging from 3 to 23 l/hour m², preferably from 8 to 18 l/hour m², even more preferably from 10 to 14 l/hour m².
The lyophilization step d) of the filtrate can be carried out at a
temperature ranging from -100 to -60 °C, preferably at -80 °C, and at a
pressure ranging from 0.01x10^{-3} mbar a 10x10^{-3} mbar, preferably from
0.1x10^{-3} mbar a 5x10^{-3} mbar, even more preferably at 1x10^{-3} mbar.
Alternatively to lyophilization, it can be used the “spray-drying” technique
to allow the removal of water. In this case, the liquid obtained from
reversed osmosis is subjected to a desiccation process by means of
spraying (nebulization) at a temperature ranging from 100 and 250 °C,
preferably from 130 and 200 °C, at a pressure ranging from 100 and 250
mmHg, preferably from 130 and 200 mmHg, for a time duration ranging
from 0.05 and 5 seconds, preferably from 0.1 and 1.5 seconds.

The purification e) step of C-3-G can be carried out by means of
reversed phase chromatography HPLC, for example by using an ODS C-18
column, 250 mm length and internal diameter of 4.6 mm.

The HPLC purification can be performed by using a step
gradient with the aid of two eluents, respectively, eluent A containing a
volatile organic acid in a concentration ranging from 25 to 250 ml/l,
preferably from 50 and 200 ml/l, even more preferably 100 ml/l and water
to complete the 1 liter volume (respectively, between 975 and 750 ml/l,
more preferably between 950 and 800 ml/l, even more preferably 900 ml/l)
and eluent B containing a volatile organic acid in a concentration ranging
from 10 to 100 ml/l, preferably from 25 to 75 ml/l, even more preferably 50
ml/l, a primary, secondary or tertiary alcohol in concentration ranging from
300 to 800 ml/l, preferably from 400 to 700 ml/l, even more preferably 500
ml/l, and water to complete the volume of 1 liter, the step gradient being
obtained as follows: isocratic elution with 100% of eluent A of a minimal
duration of 1 minute and maximal of 15 minutes; from 0.5 to 5 minutes up
to reach a percent of eluent A ranging from 95% and 80%; from 5 to 50
minutes up to reach a percent of eluent A ranging from 50% and 0%. The
flow of the chromatographic run is kept constant at a rate ranging from 0.8
and 1.8 ml/min, preferably at a rate ranging from 1.0 and 1.5 ml/min, even
more preferably at a rate of 1.2 ml/min. The temperature too is kept
constant at values ranging from 0 and 30 °C, preferably from 8 and 25 °C,
even more preferably at 23 °C. The volatile organic acid can be chosen in
the group consisting of formic acid, malonic acid, ossalic acid, succinic
acid, trifluoroacetic acid, trichloroacetic acid, acetic acid, while the alcohol
can be selected in the group consisting of methanol, ethanol, n-butanol,
sec-butylic alcohol, tert-butylic alcohol, propanol, isopropanol. The spectrophotometric detection can be carried out by means of a diode array UV-visible detector set up for the acquisition of the chromatographic runs between 200 and 600 nm wavelength, particularly at the wavelength of 515 nm, or by means of a fixed wavelength UV-visible detector and set up for the acquisition at a wavelength of 515 nm, or by means of a spectrometric mass detector set up for the detection of primary ions with a molecular weight of 449 and 535 atomic mass unit (a.m.u.).

The method according to the invention can comprise a further step f) of evaporation and condensation of the solution obtained after the e) step of purification.

It represents a further object of the present invention an extract enriched in C-3-G and of its derivatives free of toxic compounds and containing at least from 50 to 95%, preferably 70-93%, even more preferably 90% of C-3-G and its derivatives, for example cyanidin-3-O-β-glucopyranoside and/or malonylated cyanidin-3-O-β-glucopyranoside, percent calculated with respect to the content present in the starting juice, and obtainable according to the method as afore defined comprising the steps a), b) and c) and/or d). This extract can further contain vitamin C (ascorbic acid).

The extract according the invention can advantageously be employed in the medical setting, therefore it represents a further object of the present invention a formulation containing as the active compound the extract as afore defined together with at least an additive and/or and adjuvant pharmaceutically acceptable. In addition, the invention concerns the use of the extract as afore defined for the preparation of a dietary supplement (nutraceutical) or of an antioxidant preparation, an antiaging preparation, a preparation with differentiating activity towards tumoral cells, with protective activity towards post-ischemic tissues.

Moreover, it is also a further object of the present invention a dietary supplement (nutraceutical) containing the extract of the invention.

The method according to the invention also allows the purification of cyanidin-3-O-β-glucopyranoside and malonylated cyanidin-3-O-β-glucopyranoside at a purity degree of about the 99%, which can be therefore advantageously utilized for the preparation of pharmaceutical products composed by cyanidin-3-O-β-glucopyranoside, or malonylated cyanidin-3-O-β-glucopyranoside or cyanidin-3-O-β-glucopyranoside +
malonylated cyanidin-3-O-β-glucopyranoside in variable proportions, to be used for the therapeutic indications that takes advantage of the known biological properties of the compounds containing cyanidin (antioxidant, antiaging, rheological, differentiating of the tumoral cells, protective of the post-ischemic tissues, etc.).

The present invention will now be described for illustrative, but not limitative, purposes according to its preferred realization forms, with particular reference to the attached figures and draws, in which:

Figure 1 shows the chromatographic trace at 515 nm wavelength of the lyophilized filtrate, resuspended in water and subjected to reversed-phase HPLC chromatography as described in the Example 1, in which are well distinguishable 2 main peaks having retention times of 12.11 and 15.08 minutes. These retention times correspond to standard C-3-G and standard malonylated C-3-G, respectively.

Figure 2 illustrates the absorbance spectrum of the peak of Figure 1 having a retention time of 12.11 minutes which shows two maxima of absorbance at 275 and 515 nm wavelength (this last in the spectral region characteristic of cyanidin-containing compounds) overlappable to that of standard C-3-G.

Figure 3 shows the absorbance spectrum of the peak of Figure 1 having a retention time of 15.08 minutes which shows two maxima of absorbance at 275 and 515 nm wavelength (this last in the spectral region characteristic of cyanidin-containing compounds) overlappable to that of standard malonylated C-3-G.

Figure 4 illustrates the mass spectrum, obtained by using an ion-trap mass spectrometer for HPLC, connected in parallel to the spectrophotometric detector, which indicates a molecular weight of 449 Da for the peak of Figure 1 with a retention time = 12.11 min, i.e. the molecular weight of C-3-G.

Figure 5 illustrates the mass spectrum, obtained by using an ion-trap mass spectrometer for HPLC, connected in parallel to the spectrophotometric detector, which indicates a molecular weight of 535 Da for the peak of Figure 1 with a retention time = 15.08 min, i.e. the molecular weight of malonylated C-3-G.

Figure 6 shows the protective effect of increasing concentrations of the peaks of C-3-G and malonylated C-3-G purified from blood orange juice towards the peroxidation of human LDL induced by
copper ions. The two curves of protection are overlappable indicating an equal antioxidant potency of the two compounds, potency which has not been compromised by the purification process.

**Example 1**: *Extraction of cyanidin according to the method of the invention and analysis of the extract.*

The purification procedure of the C-3-G, with the aim of obtaining the molecule at a purity degree suitable to prepare pharmaceutical products based on C-3-G, has been set up in the laboratory setting and needs of the proper scale up to go to the pilot plant level and subsequently to the industrial process production.

The laboratory procedure has been carried out by using two liters of the juice of blood oranges of Sicily, containing 1.1. mmoles of total cyanidins/l of juice and 3 mmoles of ascorbic acid/l of juice. The juice has been centrifuged (5000 rpm for 15 minutes at 4 °C) in order to remove all the particulate matter formed during the squeezing process. The resulting supernatant has been subjected to a process of reversed osmosis using a cartridge containing a polymeric membrane with tangential flow and at wrapped spiral made of cellulose acetate (having a molecular filtration cut-off for uncharged solutes of 300 Da). The wrapped spiral configuration has been chosen for its efficiency and economy, highly suitable and ideal for an industrial process production in which large amounts of liquid have to be filtered with the maximal effectiveness, in the shortest time possible and at the lowest cost possible. The blood orange juice to be treated has been placed under pressure on one side of the filtering system at the maximal operating conditions as indicated by the suppliers of the filtering system (27 bar pressure, 10-14 l/h m² permeability). As a consequence of the radial component of the pressure, part of the liquid containing the non-ionic solutes with a molecular weight equal to or lower than 300 Da has permeate through the membrane whilst, due to the tangential component of the pressure, the non-permeated liquid, containing the ionic solutes having a weight equal to or higher than 300 Da, has been driven through the outlet of the membrane.

The filtrate so obtained has been then analyzed to determine the content in C-3-G and ascorbic acid and to allow to calculate the yield of the process of reversed osmosis. To this purpose, an aliquot corresponding to 200 µl has been assayed by ion-pairing HPLC for the determination of the ascorbic acid concentration (20) and 200 more µl
have been analyzed by the direct spectrophotometric assay (4) to evaluate the content in total cyanidins. For the determination of ascorbic acid concentration, an HPLC system with the low pressure mixing of the solvents has been used, the system being equipped with a diode array UV-visible spectrophotometric detector with a 5 cm light-path flow-cell which increases the sensitivity of the detecting system, whilst the determination of total cyanidin content a single-ray Beckman DU-7 spectrophotometer has been used. The analyses allowed to reveal a concentration of 1.0 mmole of total cyanidins/l of filtrate and 1.5 mmole of ascorbic acid/l of filtrate. On the basis of these results, the analysis of the permeate, which should have contained the non-ionic solutes with a molecular weight equal to or lower than 300 Da, has also been performed. The results of these analyses showed traces lower than 0.1 mmole of total cyanidins and an amount of ascorbic acid equal to that found in the filtrate (1.5 moles/l). As it could be expected, at up to this step of the purification process, the relative yield of the total cyanidins has been equal to about the 91% of the initial amount of cyanidins whilst, for ascorbic acid, the yield has been equal to 50%. This last result has to be attributed to the molecular weight of ascorbic acid which is lower than the molecular weight cut-off of the membrane utilized. Only the net charge possessed by ascorbic acid allowed to obtain in the filtrate also a significant amount of this natural antioxidant.

The subsequent step has been represented by a lyophilization process of the filtrate. To this purpose, the filtrate (about one liter of solution containing 1 mmole of total cyanidins and 1.5 mmole of ascorbic acid) has been frozen at -80 °C and subsequently connected to a laboratory lyophilizer device Edwards with a freezing plate by -80 °C and a depressurization of the lyophilization room at 1 x 10⁻³ mbar. An aliquot of the lyophilized product, highly water-soluble, has been dissolved in water in order to have a theoretical cyanadin concentration of 0.1 mM. This solution has been subjected to an analytical separation on a reversed phase HPLC column ODS C-18, with 5 μm particle size, 250 mm length and internal diameter of 4.6 mm. The HPLC apparatus used has been afore described and the C-3-G separation has been performed by a step gradient using two eluents having the following composition: eluent A (1 liter), 100 ml of HCOOH and 900 ml of H₂O; eluent B (1 liter), 50 ml of HCOOH, 450 ml of water, 500 ml of CH₃OH. The step gradient was as
follows: 3 minutes of isocratic elution with 100% eluent A; 1 minute at up to 90% of eluent A; 16 minutes at up to 0% of eluent A. The flow of the chromatographic run has been kept constant at 1.2 ml/min and the temperature at 23 °C. The diode-array spectrophotometric detector has been set up for the data acquisition between 200 and 600 nm wavelength. Under these chromatographic conditions, at 515 nm wavelength (characteristic of the cyanidin-containing compounds) two main peaks were resolved having the retention time and the spectrum of absorbance of standard C-3-G (the one with the retention time of about 12 minutes) and of standard malonylated C-3-G (the one with the retention time of about 15 minutes), respectively (Figure 1).

As far as the peak with the retention time of about 12 minutes is concerned (12.11 minutes), the spectrum of absorbance showed two maxima of absorbance, one at 275 and one at 515 nm wavelength, this last in the spectral region characteristic of the cyanidin-containing compounds, and it was overlappable to that of standard C-3-G (Fig. 2).

As far as the peak with the retention time of about 15 minutes is concerned (15.08 minutes), the spectrum of absorbance showed two maxima of absorbance, one at 280 and one at 520 nm wavelength, this last in the spectral region characteristic of the cyanidin-containing compounds, and it was overlappable to that of standard malonylated C-3-G (Fig. 3).

The analysis of the mass spectrum, obtained by using an ion-trap HPLC mass spectrometer, connected in parallel to the spectrophotometric detector, has indicated for the peak of Figure 1 with the retention time = 12.11 min a molecular weight of 449 Da, i.e. the molecular weight of C-3-G (Figure 4).

The analysis of the mass spectrum, obtained by using an ion-trap HPLC mass spectrometer, connected in parallel to the spectrophotometric detector, has indicated for the peak of Figure 1 with the retention time = 15.08 min a molecular weight of 535 Da, i.e. the molecular weight of malonylated C-3-G (Figure 5).

The respective concentrations in the lyophilized filtrate of C-3-G and malonylated C-3-G have been equal to 0.6 and 0.4 mmoles, respectively. It should be underlined that the lyophilized filtrate also contained the 50% of the initial content of ascorbic acid, i.e.: about 1.6 mmoles. The residual lyophilized filtrate has been resuspended in water
and subjected to reversed phase HPLC under the conditions afore described. The two peaks of C-3-G and malonylated C-3-G were separately collected. A purity level for both peaks of about 99% (based on the absorbance and mass spectra) was obtained.

It is worth recalling that the below reported formulas can be used to optimize the chromatographic process to scale up the process to the industrial production:

\[
\text{Scale-up factor} = \frac{(\text{diameter preparative col.})^2 \times \text{length preparative col.}}{(\text{diameter analytical col.})^2 \times \text{length analytical col.}}
\]

\[
\text{Range prep flow} = \text{range analytical flow} \times \frac{(\text{diameter preparative col.})^2}{(\text{diameter analytical col.})^2}
\]

\[
\text{Duration of the Prep Grad.} = \frac{\text{length preparative col.}}{(\text{diam. Prep. col.})^2} \times \frac{\text{range anal. flow}}{\text{range prep flow}}
\]

\[
\text{Duration nalytical grad.} = \frac{\text{length analytical col.}}{(\text{diam. anal. col.})^2} \times \frac{\text{range prep flow}}{\text{range prep flow}}
\]

In Table 1, some examples are reported.

<table>
<thead>
<tr>
<th>Column diameter (mm)</th>
<th>Scale-up factor</th>
<th>Typical amount of solute dissolved in the injected sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.6</td>
<td>10</td>
<td>1 - 4 mg</td>
</tr>
<tr>
<td>10.0</td>
<td>4.7</td>
<td>5 - 25 mg</td>
</tr>
<tr>
<td>21.4</td>
<td>21.6</td>
<td>22 - 88 mg</td>
</tr>
<tr>
<td>41.4</td>
<td>81.0</td>
<td>81 - 324 mg</td>
</tr>
<tr>
<td>77.0</td>
<td>280.0</td>
<td>287 - 1150 mg</td>
</tr>
<tr>
<td>100.0</td>
<td>472.0</td>
<td>0.47 - 1.89 g</td>
</tr>
<tr>
<td>150.0</td>
<td>1,060.0</td>
<td>1.06 - 4.24 g</td>
</tr>
</tbody>
</table>
Subsequently, repeated chromatographic runs of the resuspended lyophilized filtrate have been performed, taking care during each run to separately collect the elution volumes of the peaks corresponding to C-3-G and malonylated C-3-G.

At the level of the industrial process production, due to the use of the aforementioned substance necessary in the chromatographic step of C-3-G and malonylated C-3-G purification, the removal and synchronous recovery of the methanol/formic acid solution is both mandatory and convenient. For this reason, the evaporation and condensation of the solution is needed under a temperature and pressure values in such ranges to avoid the degradation of the active substance. The evaporation of the methanol/formic acid solution is also necessary to eliminate any type of contamination that might decrease the safety and absorbability of the final product.

Therefore, semi-automatic system for the evaporation and subsequent condensation might be used. The system might also be fully automated and inserted within an industrial process of production. The system might operate at temperatures ranging from 25 and 35 °C under a vacuum pressure ranging from 0.05 to 1 mbar, these values guaranteeing the full distillation of the methanol/formic acid mixture with a recovery of about the 97-98.5% and a modest water content of about 2-3%.

The distillation capacity of methanol at 25 °C, with a load of about 10 l, is of about 2-2.5 l/h. The methanol/formic acid distillate so obtained, can be recycled for the preparation of eluents to be used in the chromatographic purification process of C-3-G.

It is important to underline that the distillation process of the methanol/formic acid mixture, can be interrupted when at least the 60-70% elimination of the initial amount of the solvents is reached, since the remaining quantity of both compounds is removable during the lyophilization step necessary to have the final preparation, stable and purified, of C-3-G or of the C-3-G + malonylated C-3-G mixture.

In the case of the described laboratory experience, both volumes of the collected peaks have been initially and separately subjected to an under vacuum evaporation process at 25 °C with a Büchi Rotavapor system, frozen at -80 °C and then again lyophilized and resuspended with such a water volume to have a final concentration of 1
mM for both compounds. Aliquots of these solutions containing C-3-G and malonylated C-3-G have been used to evaluate the biological “integrity” of the purified molecules, through a test aimed to determine their antioxidant power. To this purpose, human purified LDL have been resuspended in a proper saline buffer and exhaustively dialyzed. The LDL suspension has been divided in different aliquots each of them was supplemented with various C-3-G or malonylated C-3-G concentrations (1, 2, 5, 10, 20, 50, 100 e 200 μM) purified as previously described. LDL incubated with no addition of any antioxidant were used as controls.

The different suspensions have been subjected to oxidizing conditions caused by the addition of 40 μM Cu²⁺ and incubated for 24 hours at 37 °C, at the end of which the level of lipid peroxidation of LDL has been evaluated by measuring the amount of malondialdehyde (MDA) produced.

To determine MDA, samples withdrawn from the various incubation mixtures have been treated with acetonitrile to precipitate proteins and then extracted with ultrapure chloroform for the removal of the hydrophobic compounds. After three repeated extraction with chloroform, the aqueous phase, containing all the MDA generated during the peroxidation process of LDL induced by copper ions, has been withdrawn. Aliquots of the aqueous phase so obtained have been analyzed by HPLC for the direct determination of MDA. The results reported in Figure 6 clearly demonstrate that the procedure adopted for the extraction and purification of C-3-G from blood orange juice did not alter either the chemical structure (for example, through the loss of the glycosidic residue with the formation of the corresponding aglycone that was not revealed in detectable amount in the various chromatographic assays effected during the different steps of the purification procedure) or cause the loss of its biological properties of a molecule with very high antioxidant potency (for example, through an oxidation process that might have occur during the purification process). Furthermore, data of Figure 6 also indicate that C-3-G and malonylated C-3-G have the same antioxidant effectiveness thus allowing to hypothesize the possibility to realize a pharmaceutical product composed by C-3-G and malonylated C-3-G both purified adopting the afore described procedure.

It is important to underline that, by subjecting the blood orange juice to the purification steps here described, it is possible to stop the
purification process at the end of the first lyophilization of the filtrate obtained after the reversed osmosis or ultrafiltration/nanofiltration procedure. In such a way, it is possible to obtain a lyophilized product enriched either in C-3-G (which represents about the 60% of its total cyanidin content), or in malonylated C-3-G (which represents about the 40% of its total cyanidin content), or in ascorbic acid in a concentration equal to about the 50% of that present in the starting juice, this lyophilized product having certainly a potential commercial relevance as dietary supplement (nutraceutical) composed of anthocyanins and vitamin C. It is also worth mentioning that in this extract the C-3-G and its malonylated derivative have a concentration equal to about the 90% of the concentration present in the starting juice.

It is also important to underline that, by submitting the blood orange juice to the purification steps here described, it is possible to process the filtrate, obtained after the reversed osmosis or ultrafiltration/nanofiltration procedure, directly to the chromatographic purification step of C-3-G and malonylated C-3-G, thus optimizing the time and yield of the purification process of the two compounds at a purity degree of about the 99%, both usable for the preparation of pharmaceutical products having as active drugs C-3-G, malonylated C-3-G or a combination, in equal or different percentages, of C-3-G and malonylated C-3-G.

REFERENCES


1. Method for the preparation of an extract enriched in cyanidin-3-O-β-glucopyranoside and its derivatives from fruits or vegetables containing these compounds, the method comprising the following steps:
   a) collection of the fruits or vegetables and removal of the possible presence of particulate matter;
   b) filtration of the liquid obtained in step a) by using reversed osmosis or ultrafiltration/nanofiltration which use a membrane with a molecular weight cut-off to filtrate uncharged solutes ranging from 100 to 800 Da; and
   c) collection of the filtrate.
2. Method according to claim 1, in which the membrane has a molecular weight cut-off ranging from 200 to 500 Da.
3. Method according to anyone of the previous claims, in which the membrane has a molecular weight cut-off of 300 Da.
4. Method according to anyone of the previous claims, which further comprises the step d) of the lyophilization of the filtrate.
5. Method according to anyone of the previous claims, which further comprises the e) step of purification of the cyanidin-3-O-β-glucopyranoside and of its derivatives subsequently or alternatively to step c).
6. Method according to anyone of the previous claims in which the fruits are chosen in the group consisting of blood orange, strawberry, blueberry, blackberry, cherries, black mulberry, raspberry, water melon, black grape, strawberry grape.
7. Method according to anyone of the previous claims in which the vegetables are chosen in the group consisting of rhubarb, red turnip, red onion, red rice.
8. Method according to anyone of the previous claims in which the step of juice collection from fruits or vegetables is performed by squeezing.
9. Method according to anyone of the previous claims in which the removal of the possible presence of particulate matter is carried out by means of centrifugation or simple filtration through a filtering device made of inert material.
10. Method according to claim 9, in which the inert material is
selected in the group consisting of porous glass, filtering gauze, filtering paper.

11. Method according to anyone of the previous claims in which
the membrane for the reversed osmosis is polymeric.

12. Method according to claim 11, in which the polymer of the
membrane is chosen in the group consisting of cellulose acetate, polyamide, polyethersulfone.

13. Method according to anyone of the previous claims in which
the polymeric membrane for the reversed osmosis is chosen in the group
consisting of tangential-flow wrapped-spiral membrane, hollow fiber membrane.

14. Method according to anyone of the previous claims in which
the filtration is carried out at a pressure ranging from 5 to 40 bar and at a
permeability rate ranging from 3 to 23 liters/hours m².

15. Method according to claim 14, in which the filtration is
carried out at a pressure ranging from 15 to 35 bar and at a permeability
rate ranging from 8 to 18 liters/hours m².

16. Method according to anyone of the previous claims in which
the filtration is carried out at a pressure of 27 bar and at a permeability
rate ranging from 10 to 14 liters/hours m².

17. Method according to anyone of the previous claims in which
the step d) of the lyophilization of the filtrate is carried out at a temperature
ranging from -100 to -60 °C and at a pressure ranging from 0.01x10⁻³ mbar
to 10x10⁻³ mbar.

18. Method according to claim 17, in which the step d) of the
lyophilization of the filtrate is carried out at a pressure ranging from 0.1x10⁻⁴ mbar to 5x10⁻³ mbar.

19. Method according to anyone of the previous claims, in
which the step d) of the lyophilization of the filtrate is carried out at -80 °C
and at 1x10⁻³ mbar.

20. Method according to anyone of the previous claims in which
alternatively to the step d) of lyophilization, the removal of water from the
filtrate is carried out by means of the "spray-drying" technique in which the
liquid obtained by the reversed osmosis is subjected to a desiccation
process by nebulization at a temperature ranging from 100 and 250 °C,
preferably from 130 and 200 °C, at a pressure ranging from 100 and 250
mmHg, preferably from 130 and 200 mmHg, for a duration time varying from 0.05 and 5 seconds, preferably from 0.1 and 1.5 seconds.

21. Method according to any of the previous claims, in which the purification of the cyanidin is carried out by reversed phase HPLC.

22. Method according to claim 21, in which the HPLC is performed using a reversed phase column ODS C-18 with 5 μm particle size, 250 mm length and 4.6 mm internal diameter.

23. Method according to any of the previous claims in which the reversed phase HPLC chromatography is performed using a step gradient with two eluents, respectively, eluent A containing a volatile organic acid in a concentration ranging from 25 and 250 ml/l and water to complete the 1 liter volume, and eluent B containing a volatile organic acid in a concentration ranging from 10 and 100 ml/l, a primary, secondary or tertiary alcohol in a concentration ranging from 300 to 800 ml/l and water to complete the 1 liter volume; the step gradient is formed as follows: isocratic elution with 100% of eluent A of a minimal length of 1 minute and maximal of 15 minutes; from 0.5 to 5 minutes at up to have a percent of eluent A ranging from 95% to 80%; from 5 to 50 minutes at up to have a percent of eluent A ranging from 50% to 0%; the flow of the chromatographic run is kept constant at a rate ranging from 0.8 and 1.8 ml/min; the temperature too is kept constant at values ranging from 0 to 30 °C.

24. Method according to claim 23, in which the volatile organic acid in the eluent A is in the concentration ranging from 50 to 200 ml/l.

25. Method according to claim 24, in which the volatile organic acid in the eluent A is in the concentration of 100 ml/l.

26. Method according to claim 23, in which the volatile organic acid in the eluent B is in the concentration ranging from 25 to 75 ml/l.

27. Method according to claim 26, in which the volatile organic acid in the eluent B is in the concentration of 50 ml/l.

28. Method according to claim 23, in which the alcohol in the eluent B is in the concentration ranging from 400 to 700 ml/l.

29. Method according to claim 28, in which the alcohol in the eluent B is in the concentration of 500 ml/l.

30. Method according to claim 23, in which the flow is at a rate ranging from 1.0 and 1.5 ml/l.
31. Method according to claim 30, in which the flow is at a rate of 1.2 ml/l.
32. Method according to claim 23, in which the temperature is ranging from 8 to 25 °C.
33. Method according to claim 32, in which the temperature is 23 °C.
34. Method according to claim 23, in which the volatile organic acid is chosen in the group consisting of formic acid, malonic acid, ossalic acid, succinic acid, trifluoroacetic acid, trichloroacetic acid, acetic acid.
35. Method according to claim 23, in which the alcohol is chosen in the group consisting of methanol, ethanol, n-butanol, sec-butyl alcohol, tert-butyl alcohol, propanol, isopropanol.
36. Method according to anyone of the previous claims in which the spectrophotometric detection is carried out by using a diode array detector set up for the acquisition of the chromatographic runs between 200 and 600 nm wavelength, or by means of a fixed wavelength UV-visible detector or by means of a mass spectrometric detector set up for the acquisition of primary ions of a molecular weight of 449 and 535 atomic mass units (a.m.u.).
37. Method according to claim 36, in which the detection is carried out at 515 nm wavelength.
38. Method according to anyone of the previous claims which further comprises a step f) of evaporation and condensation of the solution obtained after the step e) of purification.
39. Extract enriched in cyanidin free of any toxic compounds containing at least from the 50 to the 95% of the initial content present in the juice of cyanidin-3-O-β-glucopyranoside and of its derivatives, obtainable by using the method as defined in anyone of the claims 1-3 and optionally 4.
40. Extract according to claim 39, comprising at least from the 70 to the 93% of the initial content present in the juice of cyanidin-3-O-β-glucopyranoside and of its derivatives.
41. Extract according to claim 39, comprising at least the 90% of the initial content present in the juice of cyanidin-3-O-β-glucopyranoside and of its derivatives.
42. Extract according to anyone of the claims 39-41, which further comprises vitamin C (ascorbic acid).
43. Extract according to anyone of the claims 39-42, in which the cyanidin is selected in the group consisting of cyanidin-3-O-β-glucopyranoside and malonylated cyanidin-3-O-β-glucopyranoside.

44. Extract according to anyone of the claims 39-43, for the use in the medical setting.

45. Composition comprising as active drug the extract as defined in the claims 39-43 together with at least an excipient and/or an adjuvant pharmaceutically acceptable.

46. Dietary supplement comprising of the extract as defined in the claims 39-43.

47. Use of the extract as defined in the claims 39-43 for the preparation of a dietary supplement.

48. Use of the extract as defined in the claims 39-43 for the preparation of an antioxidant medicine.

49. Use of the extract as defined in the claims 39-43 for the preparation of an antiaging medicine.

50. Use of the extract as defined in the claims 39-43 for the preparation of a medicine having differentiating activity towards tumoral cells.

51. Use of the extract as defined in the claims 39-43 for the preparation of a medicine having protective activity towards ischemia and reperfusion tissue damages.
Fig. 2
Fig. 5
Fig. 6