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(54) Title: HIGH-PURITY RUBUSOSIDE AND PROCESS FOR PRODUCING OF THE SAME

(57) Abstract: The invention provides a process of producing Rubusoside from steviol glycosides of *Stevia rebaudiana* plant. The process is useful for producing high purity Rubusoside with purity greater than 95% (dry basis). High purity rubusoside is useful as in combination with other caloric and non-caloric sweeteners as well as non-caloric sweetener in various food and beverage compositions. The high purity rubusoside is useful as non-caloric sweetener in edible and chewable compositions such as any beverages, confectionaries, bakeries, cookies, chewing gums, and alike.



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HIGH-PURITY RUBUSOSIDE AND PROCESS FOR PRODUCING OF THE SAME

This application is continuation in part for US Pat. Application (provisional) ser. No. 61/522,237, filed on 10 August 2011.

Field of the Invention

[0001] The invention relates to a process for producing steviol glycosides, more particularly Rubusoside from steviol glycosides of *Stevia rebaudiana* plant.

Background of the Invention

[0002] Nowadays high intensity sweeteners are used worldwide. They can be of both synthetic and natural origin.

[0003] Non-limiting examples of synthetic sweeteners include sucralose, potassium acesulfame, aspartame, alitame, saccharin, neohesperidin dihydrochalcone synthetic derivatives, cyclamate, neotame, dulcin, suosan, N-[N-[3-(3-hydroxy-4-methoxyphenyl)propyl]-L- α -aspartyl]-L-phenylalanine 1-methyl ester, N-[N-[3-(3-hydroxy-4-methoxyphenyl)-3-methylbutyl]-L- α -aspartyl]-L-phenylalanine 1-methyl ester, N-[N-[3-(3-methoxy-4-hydroxyphenyl)propyl]-L- α -aspartyl]-L-phenylalanine 1-methyl ester, salts thereof, and the like.

[0004] Non-limiting examples of natural high intensity sweeteners include Stevioside, Rebaudioside A, Rebaudioside B, Rebaudioside C, Rebaudioside E, Rebaudioside F, Steviolbioside, Dulcoside A, Rubusoside, mogrosides, brazzein, neohesperidin dihydrochalcone (NHDC), glycyrrhizic acid and its salts, thaumatin, perillartine, pernandulcin, mukuroziosides, baiyunoside, phlomisioside-I, dimethyl-hexahydrofluorene-dicarboxylic acid, abrusosides, periandrin, carnosiflosides, cyclocarioside, pterocaryosides, polypodoside A, brazilin, hernandulcin, phillodulcin, glycyphyllin, phlorizin, trilobatin, dihydroflavonol, dihydroquercetin-3-acetate, neoastilbin, *trans*-cinnamaldehyde, monatin and its salts, selligueain A, hematoxylin, monellin, osladin,

pterocaryoside A, pterocaryoside B, mabinlin, pentadin, miraculin, curculin, neoculin, chlorogenic acid, cynarin, siamenoside and others.

[0005] *Stevia rebaudiana* Bertoni is a perennial shrub of the *Asteraceae* (*Compositae*) family native to certain regions of South America. The leaves of the plant contain from 10 to 20% of diterpene glycosides, which are around 150 to 450 times sweeter than sugar. The leaves have been traditionally used for hundreds of years in Paraguay and Brazil to sweeten local teas and medicines.

[0006] The extract of *Stevia rebaudiana* plant contains a mixture of different sweet diterpene glycosides, which have a single base – steviol and differ by the presence of carbohydrate residues at positions C13 and C19. These glycosides accumulate in *Stevia* leaves and compose approximately 10% - 20% of the total dry weight. Typically, on a dry weight basis, the four major glycosides found in the leaves of *Stevia* are Dulcoside A (0.3%), Rebaudioside C (0.6-1.0%), Rebaudioside A (3.8%) and Stevioside (9.1%). Other glycosides identified in *Stevia* extract include Rebaudioside B, D, E, and F, Steviolbioside and Rubusoside. Among steviol glycosides only Stevioside and Rebaudioside A are available in commercial scale.

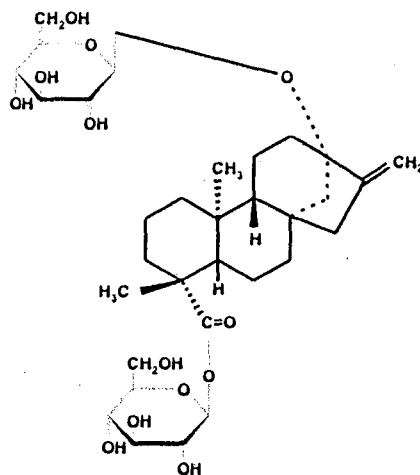
[0007] The physical and sensory properties are well studied only for Stevioside and Rebaudioside A. The sweetness potency of Stevioside is around 210 times higher than sucrose, Rebaudioside A in between 200 and 400 times.

[0008] On the other hand commercial preparations of steviol glycosides such as *Stevia* Extract, Rebaudioside A possess certain drawbacks substantially limiting their usage in mainstream products.

[0009] It has to be noted that high intensity sweeteners' taste profile is highly dependant on the concentration and usually the higher the concentration the higher the sensation of undesirable taste components such as bitterness, licorice, lingering aftertaste. This phenomenon limits the usage of steviol glycosides further to 4-5% sucrose equivalents in order to achieve pleasant taste of a food or beverage sweetened with *stevia* sweeteners.

[0010] Therefore in many cases various sweeteners are used in blends to benefit from the effect of synergism, which allows the usage of sweeteners at lower concentrations where undesirable taste profile attributes are less prominent. It has to be noted that

synergistic effect can be achieved both between different high intensity sweeteners as well as between high intensity and bulk sweeteners such as sucrose etc.



RUBUSOSIDE

[0011] Rubusoside (CAS No: 64849-39-4), is one of sweet sweet glycosides found in *Stevia rebaudiana*. Its concentration in dried leaves of *Stevia* is usually <0.2%. However rubusoside is also found in leaves of *Rubus suavissimus* S. Lee (Chinese sweet leaf). Rubusoside is the main steviol glycoside found in the leaves of *Rubus suavissimus*.

[0012] Recent studies show that Rubusoside possess certain valuable properties. Particularly WIPO Patent Application WO/2011/090709 describes sweetness-enhancing properties of Rubusoside. US Patent Application 12/937,055 describe Rubusoside usage as a natural solubilizing agent for a number of compounds.

[0013] These properties multiply the significance of Rubusoside and attract great interest for processes of preparation of highly purified forms of Rubusoside.

[0014] There are few processes described in for Rubusoside preparation.

[0015] WIPO Patent Application WO/2011/090709 describes a process for preparing high purity rubusoside wherein the commercial crude Rubusoside extract (63.7% purity) was dissolved in aqueous methanol and subjected to chromatographic purification on a column packed with reverse-phase stationary phase. The fractions with high Rubusoside content were combined, dried and refluxed with methanol, to prepare Rubusoside having 94.6% purity. It has to be noted that employing chromatographic separation techniques in large scale production is not feasible and is suitable generally for Lab or pilot scale processes.

[0016] US Patent Application 12/937,055 describes a process for Rubusoside preparation wherein *Rubus suavissimus* dried leaves were extracted with water and the water extract was dried to yield a crude extract containing 5-15% rubusoside (w/w). The dried crude extract was dissolved in water and subjected to column chromatography with macroporus adsorbent. As a result Rubusoside was adsorbed on macroporous resin and subsequently eluted with ethanol to obtain a purified extract containing ca. 60% rubusoside. Subsequently, the purified extract was subjected to chromatography on a column packed with silicagel and the fractions rich in Rubusoside were dried to yield Rubusoside with ca. 80% purity. The said material was further re-crystallized from Methanol to yield rubusoside with >99% purity. As discussed above, processes utilizing chromatographic techniques are suitable for Lab or pilot scale production only.

[0017] In all above-mentioned inventions Rubusoside is derived from *Rubus suavissimus* leaf extracts. It has to be noted that the supply and availability of *Rubus suavissimus* leaves is somewhat limited.

[0018] On the other hand the extracts and purified glycosides of *Stevia rebaudiana* are relatively easy to obtain. Particularly there's a large and cheap supply of high purity Stevioside (98%) and *Stevia rebaudiana* extracts containing high levels of Stevioside (up to 80%). The cultivation of *Stevia rebaudiana* is much simpler and currently there are Stevia commercial growers in various regions of the world.

[0019] Thus it can be concluded, there is a need for a simple, efficient, and economical process for production of high purity Rubusoside from easily accessible raw materials.

SUMMARY OF THE INVENTION

[0020] The invention relates to a process for production of steviol glycosides, and more particularly to a process for production of Rubusoside.

[0021] The primary objective of the invention is to develop an efficient process of producing Rubusoside from glycosides of *Stevia rebaudiana*.

[0022] One aspect of present invention provides a process of biocatalytic conversion of Stevioside to Rubusoside. In one embodiment the process comprises the steps of:

[0023] I. providing initial steviol glycosides such as highly purified Stevioside or an extract of *Stevia rebaudiana* containing Stevioside, or a mixture of steviol glycosides one of which is Stevioside;

[0024] II. dissolving the initial steviol glycosides in the water;

[0025] III. providing an enzyme with glycosyl hydrolase activity to obtain reaction mixture;

[0026] IV. incubating the reaction mixture to facilitate complete or partial transformation of Stevioside to Rubusoside;

[0027] V. terminating the reaction by thermal inactivation of enzyme;

[0028] VI. recovering and purifying highly purified Rubusoside from reaction mixture.

[0029] Another aspect of the present invention provides a process for recovery and purification of Rubusoside from reaction mixture. In one embodiment the process comprises the steps of:

[0030] i. providing reaction mixture containing Rubusoside;

[0031] ii. contacting the reaction mixture with macroporous adsorbent resin;

[0032] iii. eluting the adsorbed Rubusoside with aqueous alcohol;

[0033] iv. removing the alcohol from eluate and drying the solution to yield a purified Rubusoside.

[0034] In yet another embodiment, the recovery and purification process comprises steps of:

[0035] a. providing reaction mixture containing Rubusoside;

[0036] b. drying the reaction mixture;

[0037] c. dissolving the extract in a first aqueous alcoholic solution to obtain a first mixture;

[0038] d. inducing crystallization in the first mixture by temperature gradient treatment;

[0039] e. filtering the first mixture to obtain a first precipitate and a first filtrate;

[0040] f. dissolving the first precipitate in a second aqueous alcoholic solution to obtain a second mixture;

[0041] g. adding activated carbon to second mixture and filtering it to obtain decolorized second mixture;

[0042] h. inducing crystallization in the decolorized second mixture by temperature gradient treatment;

[0043] i. filtering the decolorized second mixture to obtain a second precipitate and a second filtrate;

[0044] j. suspending the second precipitate in a third aqueous alcoholic solution to obtain a third mixture;

[0045] k. filtering the third mixture to obtain a third precipitate and a third filtrate; and

[0046] l. drying the third precipitate to yield purified Rubusoside.

[0047] In another embodiment of the process, the first aqueous alcoholic solution in step (c) is a methanol-water solution, with 75-99% methanol.

[0048] In another embodiment of the process, the second aqueous alcoholic solution in step (f) is a methanol-water solution with 70-90% methanol.

[0049] In another embodiment of the process, the third aqueous alcoholic solution in step (j) is a methanol-water solution with 90-99% methanol.

[0050] In another embodiment of the process, in steps (d) and (h) inducing crystallization comprises increasing the mixture temperature to 65-70°C then gradually cooling it to 10°C at a rate of 10°C per hour with continuous mild agitation.

[0051] In another embodiment of the process, the aqueous alcoholic solution comprises one or more organic solvents selected from the group consisting of methanol, ethanol, 1-propanol, and isopropanol.

[0052] In another embodiment of the process, the purified Rubusoside has purity greater than 95% on a dry basis.

[0053] In another embodiment of the process, the purified Rubusoside has purity greater than 98% on a dry basis.

[0054] Another aspect of the present invention provides a product comprising high purity Rubusoside, wherein the product is selected from the group consisting of food, beverage, pharmaceutical composition, tobacco, nutraceutical, oral hygienic composition, or cosmetic.

[0055] Another aspect of the present invention provides a sweetener composition comprising high purity Rubusoside.

[0056] In another embodiment, the sweetener composition further comprises Rebaudioside A, enzymatically modified stevia, Rebaudioside D, a mixture of steviol glycosides with more than 95% (on dry basis) total steviol glycosides content, high intensity sweetener and natural flavor compound, caloric sweetener, or sucrose.

[0057] In another embodiment, the sweetener composition further comprises one natural high intensity sweetener selected from the group consisting of: steviol glycosides including a purified sweet steviol glycoside mixture, stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, dulcoside A, dulcoside B, stevia, alpha-glucosyl stevia, fructosyl stevia, galactosyl stevia, beta-glucosyl stevia; siamenoside; mogroside IV; mogroside V; Luo Han Guo sweetener; monatin and its salts (monatin SS, RR, RS, SR); glycyrrhizic acid and its salts; curculin; thaumatin; monellin; mabinlin; brazzein; hernandulcin; phyllodulcin; glycyphyllin; phloridzin; trilobatin; baiyunoside; osladin; polypodoside A; pterocaryoside A; pterocaryoside B; mukurozioside; phlomisoside I; periandrin I; abrusoside A; cyclocarioside I; and combinations thereof.

[0058] It is to be understood that the described biocatalytic conversion process can be applied also to other steviol glycosides wherein the hydrolytic action of various glycosyl hydrolases can produce new steviol glycosides' molecules.

[0059] It is to be understood that the foregoing descriptions and specific embodiments shown herein are merely illustrative of the best mode of the invention and the principles thereof, and that modifications and additions may be easily made by those skilled in the art without departing for the spirit and scope of the invention, which is therefore understood to be limited only by the scope of the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0060] The accompanying drawings are included to provide a further understanding of the invention. The drawings illustrate embodiments of the invention and together with the description serve to explain the principles of the embodiments of the invention.

[0061] FIG. 1 shows HPLC chromatograms of reaction mixture after 0 hrs (A), 6 hrs (B) and 24 hrs (C).

DETAILED DESCRIPTION OF THE INVENTION

[0062] Advantages of the present invention will become more apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

[0063] The invention provides a process for production and purification of rubusoside.

[0064] In one embodiment of present invention, the process of the isolation and purification begins with providing Stevioside derived from *Stevia rebaudiana* extract, containing 90-100%, preferably 95-99% (on dry basis) Stevioside.

[0065] Stevioside is dissolved in water to obtain a solution with 1-50%, preferably 5-30%, more preferably 8-10% (wt/vol) concentration. The pH of the solution is adjusted to pH 3.0-8.0 preferably pH 4.5-6.5 and the temperature is maintained at 28-50°C, preferably 35-45°C. An enzyme with glycosyl hydrolase activity is added to solution to make reaction mixture. Non-limiting examples of enzymes include, rhamnosidase, β -glucosidase, hesperidinase, naringinase, pectinase, cellulase, and others, in free or immobilized forms. The reaction mixture is maintained at pH 3.0-8.0 preferably pH 4.5-6.5 and the temperature is maintained at 28-50°C, preferably 35-45°C, for about 12-24 hours, or long enough to allow the desired degree of conversion of Stevioside to Rubusoside occur. Upon completion the reaction mixture is boiled at 100°C for 10-30 min to inactivate the enzyme and then filtered with activated carbon and spray dried. Alternatively the mixture can be additionally treated with ion exchange resins, purified by

macroporous adsorption resins, membranes etc. The spray dried reaction mixture can be used "as-is" or subjected to further purification to prepare high purity Rubusoside.

[0066] For further purification the spray dried reaction mixture is admixed with a first aqueous alcoholic solution containing 70-100%, more preferably 75-99% alcohol to obtain a first mixture. The ratio (wt/vol) of spray dried reaction mixture to aqueous alcohol is 1:1 to 1:5, more preferably 1:2 to 1:4. The alcohol is selected from the group comprising ethanol, methanol, 1-propanol, 2-propanol or combinations thereof, more preferably ethanol and methanol.

[0067] In another embodiment the first mixture is incubated at a temperature 10-100°C more preferably 30-80°C for 0.5-30 min more preferably for 1-10 min.

[0068] In another embodiment the first mixture is then cooled to 0-40°C preferably 10-20°C at a rate of 8-11°C per hour, and incubated at final temperature for 1-72 hours, preferably 1-24 hours to facilitate the crystallization of Rubusoside.

[0069] In another embodiment the crystallized Rubusoside is separated from first mixture to become a first precipitate, and the remaining solution becomes a first filtrate.

[0070] In another embodiment the first precipitate has 75-99%, preferably 90-95% (on dry basis) Rubusoside content.

[0071] In another embodiment the first precipitate is admixed with a second aqueous alcoholic solution containing 60-100%, more preferably 70-90% alcohol to obtain a second mixture. The ratio (wt/vol) of first precipitate to aqueous alcohol is 1:1 to 1:5, more preferably 1:2 to 1:4. The alcohol is selected from the group comprising ethanol, methanol, 1-propanol, 2-propanol or combinations thereof, more preferably ethanol and methanol.

[0072] In another embodiment the second mixture is heated till full dissolution of first precipitate and 1-5%, preferably 1-2% of activated carbon is added and the mixture is incubated for 20 min at 60-70°C. Subsequently the activated carbon is removed by means of press filter to obtain decolorized second mixture.

[0073] In another embodiment the decolorized second mixture is incubated at a temperature 10-100°C more preferably 30-80°C for 0.5-30 min more preferably for 1-10 min.

[0074] In another embodiment the decolorized second mixture is then cooled to 0-40°C preferably 10-20°C at a rate of 8-11°C per hour, and incubated at final temperature for 1-72 hours, preferably 1-24 hours to facilitate the crystallization of Rubusoside.

[0075] In another embodiment the crystallized Rubusoside is separated from decolorized second mixture to become a second precipitate, and the remaining solution becomes a second filtrate.

[0076] In another embodiment the second precipitate has 90-100%, preferably 95-100% (on dry basis) Rubusoside content.

[0077] In another embodiment the second precipitate is further suspended in a third aqueous alcoholic solution containing 70-100%, more preferably 90-99% alcohol to obtain a third mixture. The ratio (vol/vol) of second filtrate to aqueous alcohol is 1:0 to 1:5, more preferably 1:0 to 1:2. The alcohol is selected from the group comprising ethanol, methanol, 1-propanol, 2-propanol or combinations thereof, more preferably ethanol and methanol.

[0078] In another embodiment the third mixture is then incubated at 0-40°C preferably 10-30°C for 1-144 hours, preferably 24-72 hours.

[0079] In another embodiment the third mixture is separated into a third precipitate and a third filtrate, where the third precipitate has >98% rubusoside content (on dry basis).

[0080] In another embodiment the third precipitate is dried by any means known to art to provide dry crystalline powder.

[0081] The HPLC analysis of steviol glycosides was carried out as described in FAO JECFA Monographs 10 (2010), using an Agilent Technologies (USA) "1200 series" chromatograph, equipped with Luna C18(2) 100A (Phenomenex, USA) column (4.6x250mm, 5µm), using 32:68(v/v) mixture of acetonitrile and 10 mmol/L sodium phosphate buffer (pH 2.6) as mobile phase, and UV detector at 210 nm.

[0082] The obtained rubusoside preparations can be used as sweetness enhancer, flavor enhancer and sweetener in various food and beverage products. Non-limiting examples of food and beverage products include carbonated soft drinks, ready to drink beverages, energy drinks, isotonic drinks, low-calorie drinks, zero-calorie drinks, sports drinks, teas, fruit and vegetable juices, juice drinks, dairy drinks, yoghurt drinks, alcohol beverages, powdered beverages, bakery products, cookies, biscuits, baking mixes, cereals, confectioneries, candies, toffees, chewing gum, dairy products, flavored milk, yoghurts,

flavored yoghurts, cultured milk, soy sauce and other soy base products, salad dressings, mayonnaise, vinegar, frozen-desserts, meat products, fish-meat products, bottled and canned foods, tabletop sweeteners, fruits and vegetables.

[0083] Additionally the highly purified rubusoside preparations can be used in drug or pharmaceutical preparations and cosmetics, including but not limited to toothpaste, mouthwash, cough syrup, chewable tablets, lozenges, vitamin preparations, and the like.

[0084] The highly purified rubusoside preparations can be used "as-is" or in combination with other sweeteners, flavors and food ingredients.

[0085] Non-limiting examples of sweeteners include steviol glycosides, stevioside, rebaudioside *A*, rebaudioside *B*, rebaudioside *C*, rebaudioside *D*, rebaudioside *E*, rebaudioside *F*, dulcoside *A*, steviolbioside, as well as other steviol glycosides found in *Stevia rebaudiana* Bertoni plant and mixtures thereof, stevia extract, Luo Han Guo extract, mogrosides, high-fructose corn syrup, corn syrup, invert sugar, fructooligosaccharides, inulin, inulooligosaccharides, coupling sugar, maltooligosaccharides, maltodextrins, corn syrup solids, glucose, maltose, sucrose, lactose, aspartame, saccharin, sucralose, sugar alcohols.

[0086] Non-limiting examples of flavors include lemon, orange, fruity, banana, grape, pear, pineapple, bitter almond, cola, cinnamon, sugar, cotton candy, vanilla flavors.

[0087] Non-limiting examples of other food ingredients include flavors, acidulants, organic and amino acids, coloring agents, bulking agents, modified starches, gums, texturizers, preservatives, antioxidants, emulsifiers, stabilisers, thickeners, gelling agents.

[0088] The following examples illustrate preferred embodiments of the invention.

[0089] EXAMPLE 1

[0090] Biocatalytic preparation of rubusoside from stevioside

[0091] 20 g of Stevioside extract produced by "PureCircle Sdn Bhd" (Malaysia), containing 98.1% (on dry basis) Stevioside, and 1.2% Rebaudioside *A* was dissolved in 200 mL of water and mixture was heated to 80°C and maintained for 10 min until complete dissolution. Then the mixture was cooled to 37°C and the pH was adjusted to pH 5.0. 20 Units (about 6 g) of "Hesperidinase from *Aspergillus niger*" (Sigma-Aldrich PN H8137) was added and the reaction mixture was incubated at 37°C under continuous

agitation. After 24 hrs the HPLC analysis of reaction mixture sample, showed 98% of Stevioside conversion to Rubusoside. The reaction mixture was boiled at 100°C for 15 min and then cooled down to 80°C. 2 g of activated carbon was added and the reaction mixture was incubated for 30 min at 80°C and then the carbon was separated by filtration. The obtained filtrate was evaporated under vacuum to about 30% total solids and spray dried to produce about 24g powder containing about 59.9% rubusoside (dry basis).

[0092] EXAMPLE 2

[0093] Purification of rubusoside

[0094] 10 g of spray dried reaction mixture prepared as per EXAMPLE 1 and containing 59.9% Rubusoside was dissolved in 200 mL of water and the solution was passed through a column packed with 200 mL Amberlite XAD 7HP macroporous adsorbent. The column was washed with 3 BV of water and the adsorbed Rubusoside was eluted with 300 mL 70% Ethanol. The Ethanol was evaporated and the obtained aqueous solution was dried to yield about 6 g of dry matter with 96.3% rubusoside content (dry basis).

[0095] EXAMPLE 3

[0096] Purification of rubusoside

[0097] 10 g of spray dried reaction mixture prepared as per EXAMPLE 1 and containing 59.9% Rubusoside, was dissolved in 30 mL of 98% methanol and the mixture was heated to 60°C and maintained for 10min. Then the mixture was cooled to 10°C at a rate of 10°C per hour. During the cooling the mixture was subjected to continuous moderate agitation. Starting from about 15°C fine crystals were formed. The amount of crystals subsequently increased. The mixture was incubated at 10°C during 24hrs. The crystals were separated by filtration and washed on the filter by pure methanol preliminarily chilled to 4°C. The obtained crystals were dried under vacuum at 80°C to yield about 6.1g crystals with 94.5% rubusoside content (dry basis).

[0098] EXAMPLE 4

[0099] Refining of Rubusoside

[00100] 5g of Rubusoside prepared as per EXAMPLE 3 was suspended in 1000 mL of 92% methanol at room temperature. The mixture was heated and maintained at 30°C during 48 hours. The crystals were separated by filtration and washed on the filter by pure methanol. The obtained crystals were dried under vacuum at 80°C to yield about 4.1g crystals with 98.5% rubusoside content (dry basis).

Claims

We claim:

1. A process for preparing Rubusoside from steviol glycosides of *Stevia rebaudiana* comprising the steps of:
 - a. providing initial steviol glycosides of *Stevia rebaudiana*;
 - b. dissolving the initial steviol glycosides in the water;
 - c. providing an enzyme with glycosyl hydrolase activity to obtain reaction mixture;
 - d. incubating the reaction mixture to facilitate complete or partial transformation of Stevioside to Rubusoside;
 - e. terminating the reaction by thermal inactivation of enzyme;
 - f. recovering and purifying high purity Rubusoside from reaction mixture.
2. A process of claim 1 wherein step (a) the steviol glycoside is Stevia extract, mixture of steviol glycosides or pure Stevioside.
3. A process of claim 1 wherein step (b) the concentration of dissolved steviol glycosides is 1-50% (wt/vol), preferably 5-30% (wt/vol), more preferably 8-10% (wt/vol).
4. A process of claim 1 wherein step (c) the enzyme is selected from the group of rhamnosidase, β -glucosidase, hesperidinase, naringinase, pectinase, cellulase, and other enzymes with glycosyl hydrolase activity, in free or immobilized forms.
5. A process of claim 1 wherein step (d) the molar yield of Rubusoside from Stevioside is 1-100%, preferably 80-100% and more preferably 95-100%.
6. A process of claim 1 wherein step (e) the reaction termination includes heat treatment, activated carbon treatment.
7. A process of claim 1 wherein step (f) one or more purification techniques are employed selected from the group of ion exchange treatment, macroporous adsorbent treatment, membrane process, chromatographic separation, and crystallization from various solvent systems.
8. A process of claim 1 wherein step (f) the recovery and purification of Rubusoside comprises the steps of:
 - a. providing reaction mixture containing Rubusoside;
 - b. contacting the reaction mixture with macroporous adsorbent resin;

- c. eluting the adsorbed Rubusoside with aqueous alcohol;
- d. removing the alcohol from eluate and drying the solution to yield a purified Rubusoside.

9. A process of claim 1 wherein step (f) the recovery and purification of Rubusoside comprises the steps of:

- a. providing dried reaction mixture containing Rubusoside;
 - b. dissolving the reaction mixture in a first aqueous alcoholic solution to obtain a first mixture;
 - c. inducing crystallization in the first mixture by temperature gradient treatment;
 - d. filtering the first mixture to obtain a first precipitate and a first filtrate;
 - e. dissolving the first precipitate in a second aqueous alcoholic solution to obtain a second mixture;
 - f. adding activated carbon to second mixture and filtering it to obtain decolorized second mixture;
 - g. inducing crystallization in the decolorized second mixture by temperature gradient treatment;
 - h. filtering the decolorized second mixture to obtain a second precipitate and a second filtrate;
 - i. suspending the second precipitate in a third aqueous alcoholic solution to obtain a third mixture;
 - j. filtering the third mixture to obtain a third precipitate and a third filtrate; and
 - k. drying the third precipitate to yield purified Rubusoside.
10. The process of claim 9 wherein the first aqueous alcoholic solution in step (b) is an methanol-water solution, with 75-99% methanol.
11. The process of claim 9 wherein the second aqueous alcoholic solution in step (e) is a methanol-water solution with 70-90% methanol.
12. The process of claim 9 wherein the third aqueous alcoholic solution in step (i) is an methanol-water solution with 90-99% methanol.
13. The process of claim 9 wherein steps (c) and (g) inducing crystallization comprises increasing the mixture temperature to 65-70°C then gradually

cooling it to 10°C at a rate of 10°C per hour with continuous mild agitation.

14. The process of claim 9 wherein the aqueous alcoholic solution comprises one or more organic solvents selected from the group consisting of methanol, ethanol, 1-propanol, and isopropanol.
15. The process of claim 1, wherein the purified Rubusoside has a purity greater than 95% on a dry basis.
16. The process of claim 1, wherein the purified Rubusoside has a purity greater than 98% on a dry basis.
17. A product comprising high purity rubusoside of claim 1, wherein the product is selected from the group consisting of food, beverage, pharmaceutical composition, tobacco, nutraceutical, oral hygienic composition, or cosmetic.
18. A sweetener composition comprising high purity rubusoside of claim 1.
19. The sweetener composition of claim 18, further comprising Rebaudioside *A*, enzymatically modified stevia, Rebaudioside *D*, a mixture of steviol glycosides with more than 95% (on dry basis) total steviol glycosides content, high intensity sweetener and natural flavor compound, caloric sweetener, or sucrose.
20. The sweetener composition of claim 18, further comprising one natural high intensity sweetener selected from the group consisting of: steviol glycosides including a purified sweet steviol glycoside mixture, stevioside, rebaudioside *A*, rebaudioside *B*, rebaudioside *C*, rebaudioside *D*, rebaudioside *E*, rebaudioside *F*, dulcoside *A*, dulcoside *B*, stevia, alpha-glucosyl stevia, fructosyl stevia, galactosyl stevia, beta-glucosyl stevia; siamenoside; mogroside *IV*; mogroside *V*; Luo Han Guo sweetener; monatin and its salts (monatin *SS*, *RR*, *RS*, *SR*); glycyrrhizic acid and its salts; curculin; thaumatin; monellin; mabinlin; brazzein; hernandulcin; phyllodulcin; glycyphyllin; phloridzin; trilobatin; baiyunoside; osladin; polypodoside *A*; pterocaryoside *A*; pterocaryoside *B*; mukurozioside; phlomisioside *I*; periandrin *I*; abrusoside *A*; cyclocarioside *I*; and combinations thereof.

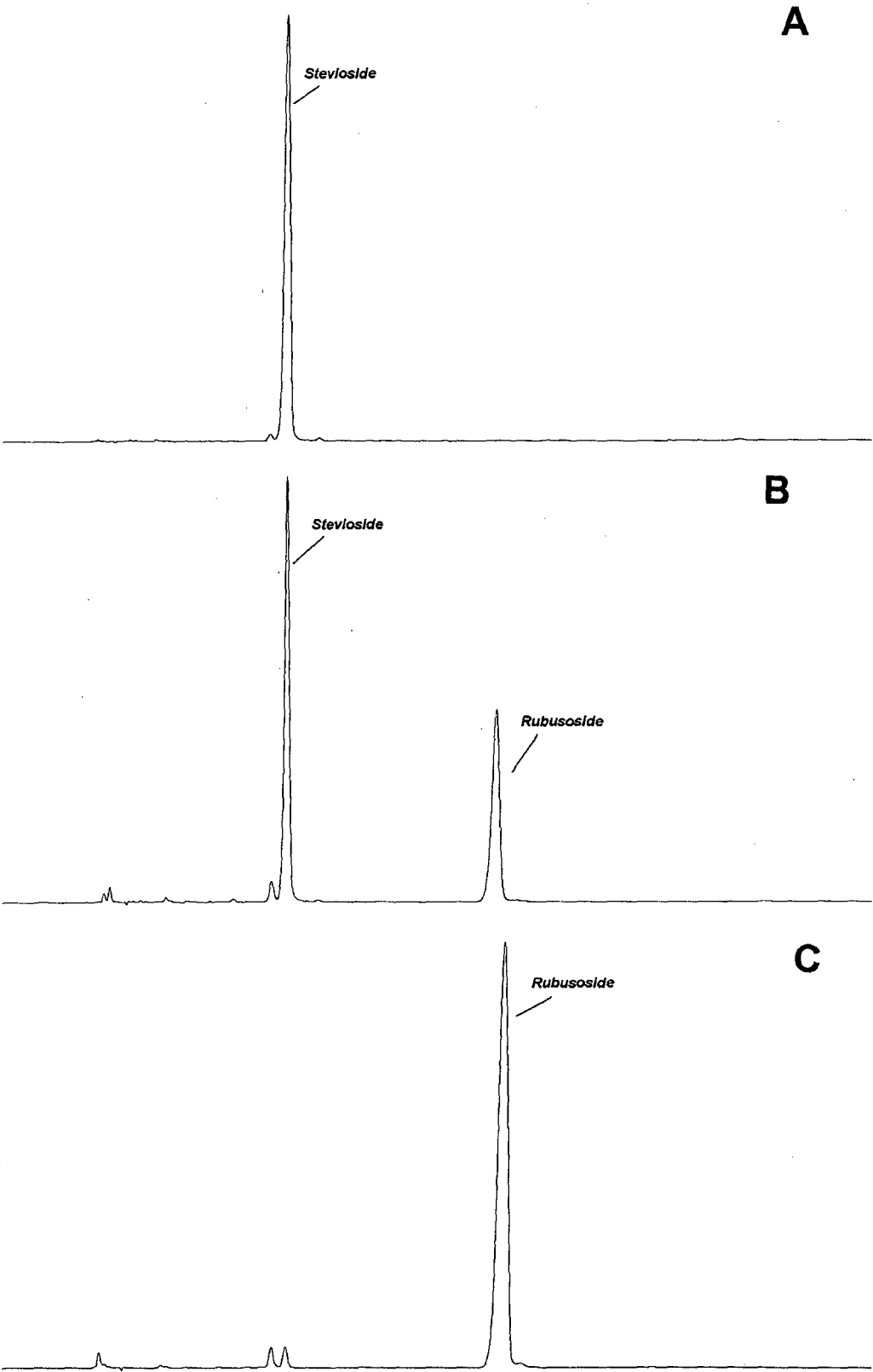


FIG 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/MY2013/000051

A. CLASSIFICATION OF SUBJECT MATTER INV. C12P19/02 C12P19/46 C12P19/56 A23L2/60 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C12P A23L		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
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☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 15 July 2013		Date of mailing of the international search report 24/07/2013
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Boeker, Ruth

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