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A METHOD FOR EXTRACTION AND PURIFICATION OF SPERMIDINE FROM ANIMAL TISSUES AND ITS APPLICATION

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Abstract

The present invention discloses a method for preparing a spermidine extract from animal muscle tissues by ultrasonication and extraction, rotary evaporation, vacuum freeze-drying and silica gel column chromatography for purification. The present invention provides a simple, convenient, fast, efficient and low-cost extraction method for extraction of natural spermidine. The method is practical and has the advantages of strong operability, short extraction time and high repeatability.

A METHOD FOR EXTRACTION AND PURIFICATION OF SPERMIDINE FROM ANIMAL TISSUES AND ITS APPLICATION

TECHNICAL FIELD

[01] The present invention belongs to a method for extraction and purification of spermidine, and relates to a method for extraction and purification of spermidine from animal muscle tissues by ultrasonication and vacuum freeze-drying and its application, which specifically comprises a method for ultrasonication and extraction of spermidine from animal muscle tissues, a method for concentration of spermidine from animal samples using a rotary evaporator combined with a vacuum freeze-dryer, and a method for purification of spermidine by using a silica gel chromatographic column.

BACKGROUND

[02] Spermidine is a low molecular weight aliphatic compound with three amino groups, and one of natural polyamines distributed widely in all living organisms. Spermidine is found in various tissues and organs in animals, such as brain, cerebellum, heart, kidney, lung and intestine. Spermidine plays an important role in regulating cell proliferation, differentiation, aging and death. Studies have shown that spermidine can reduce the infiltration of inflammatory cells, inhibit the expression of inflammatory factors and induce autophagy. In addition, spermidine has important functions such as anti-oxidation, anti-depression, anti-tumor and alleviating cerebral injury. Therefore, it is of great theoretical and practical significance to explore a method for rapid and efficient extraction of spermidine for clarifying the biological function and mechanism of action of natural spermidine in regulating animal growth and development, preventing and treating cancers.

[03] Spermidine is distributed widely in animals, plants, bacteria, fungi and other living organisms, and plays an important regulatory role in maintaining the homeostasis. Among them, the content of spermidine is higher in animal muscles and visceral tissues, soybean and fermented bean products and some mushrooms. Most of the spermidine in an organism is taken up from food, and a small part is synthesized by intestinal flora and biosynthesized by itself. Studies have shown that the levels of

polyamines in animals and humans decrease with age, but the concentration of polyamines in long-lived elderly people is close to those in young adults. Increased intake of polyamines can increase the levels of endogenous polyamines *in vivo*, and alleviate age-associated cardiovascular diseases by inducing autophagy, so as to play the role of anti-aging and prolonging life. Therefore, supplement of spermidine in the diet can help prevent diseases and prolong life. Other studies have shown that oral administration of spermidine can reduce the incidence of hepatocellular carcinoma and liver fibrosis induced by chemicals in mice, and extend the life expectancy by 25%. Studies have demonstrated that it is safe and well tolerated to supplement spermidine-rich extracts to mice and the elderly. Therefore, the use of natural spermidine extracts has broad application prospects in the field of disease prevention and anti-aging. It is of great theoretical and practical significance to explore a method for rapid and efficient extraction of spermidine.

[04] At present, there are few methods to extract spermidine from animal tissues. The present invention discloses a method for extraction and purification of spermidine from animal muscle tissues by ultrasonic extraction combined with vacuum concentration, vacuum freeze-drying and silica gel column chromatography and application thereof. In the method, a glass homogenizer is used to fully grind and crush an animal muscle tissue sample so as to release more spermidine by subsequent ultrasonic extraction. Most of spermidine in the tissue sample can be largely released after ultrasonication at 40°C for 60 min for twice. Studies have showed that it takes 2 - 3 h to extract spermidine from residues of safflower injection by reflux extraction, and the extraction is to be repeated for 3 times, with a cumulative time of 6 - 9 h. In the method, the extraction speed is improved by ultrasonication and extraction, and the temperature is more controllable, with less influence on the physicochemical properties of spermidine. Spermidine is soluble in methanol and ethanol. Ethanol has less toxic and side effects than organic solvents such as methanol and ether. Therefore, 60% ethanol (V/V) is used as a spermidine extraction solvent in the present invention. In addition, the present invention adopts rotary evaporation combined with vacuum freeze-drying, and the rotary evaporation temperature is 45°C, so that the evaporation time is shortened from 2 h to 30 min compared with evaporation at 40°C, and ethanol (methanol and chloroform) in the solvent can be basically removed; vacuum freeze-

drying is carried out at -80°C for 12 h, and a yellowish-white powder crude spermidine extract can be seen at the bottom of the freeze-dried tube after vacuum freeze-drying.

[05] By the method of the present invention, 0.1860 mg of spermidine can be extracted from 1.0 g of porcine muscle tissue, while the actual content of spermidine detected by high performance liquid chromatography (HPLC) from the same porcine muscle tissue sample is 0.2087 mg. Therefore, 89.12% spermidine can be extracted from the porcine muscle tissue using the present invention, and the yield of spermidine extracted from coumarin is about 85%. The present invention has the advantages of practical solutions, simple operation, high purity and high repeatability, and most of spermidine can be extracted from the animal muscle tissue under the ultrasonic extraction conditions; the extraction reagent is safe, controllable and low in cost; the extraction time is short with high efficiency, and a large amount of sample can be extracted per unit time. Therefore, the present invention is clearly innovative and advanced in extraction time, extraction reagent, concentration and purification.

[06] The present invention discloses a method for preparing a spermidine extract from an animal muscle tissue by ultrasonication and extraction, vacuum freeze-drying, silica gel column chromatography for purification and HPLC. The method can rapidly and efficiently extract and purify spermidine from an animal muscle tissue, and has the advantages of simple operation, good repeatability, high accuracy and short spermidine extraction period.

SUMMARY

[07] The first purpose of the present invention is to extract spermidine from animal muscle tissues by ultrasonication and extraction. The method has the advantages of simple operation, simple equipment, high repeatability and high accuracy.

[08] In order to achieve the purpose, the present invention adopts the following technical solution:

[09] Grinding and crushing an animal muscle tissue with a glass homogenizer, and extracting spermidine from the muscle tissue with 60% ethanol (V/V) as an extraction solvent; adding 60% ethanol (V/V) at a ratio of amount of tissue sample to amount of extraction solvent of 1:20 (g:mL) for ultrasonication at 40 KHz at 40°C for

60 min, followed by high speed rotary centrifugation at 12000 r/min, and collecting the supernatant; repeating the above operations to enable spermidine in the tissue to be fully dissolved in the extraction solvent to prepare a crude spermidine extract.

[010] The second purpose of the present invention is to provide a condition for concentrating a crude spermidine extract by rotary evaporation combined with vacuum freeze-drying. The method can rapidly and efficiently concentrate the crude spermidine extract with less spermidine loss, high efficiency and high repeatability.

[011] In order to achieve the purpose, the present invention adopts the following technical solution:

[012] The rotary evaporation conditions are as follows: rotary evaporation is carried out at 200 r/min at 45°C. The vacuum freeze-drying conditions are as follows: the centrifuge tube is closed with a 0.22 μm cell strainer for vacuum freeze-drying at -80°C.

[013] The third purpose of the present invention is to provide a condition for purifying a crude spermidine extract by silica gel column chromatography. The method can rapidly and efficiently purify the crude spermidine extract with high spermidine purification degree, less loss and high repeatability.

[014] In order to achieve the purpose, the present invention adopts the following technical solution:

[015] The silica gel column chromatographic conditions are as follows: a silica gel column is used, with pore size of silica gel of 60Å, 200 - 425 meshes. The freeze-dried crude spermidine extract is dissolved in 2 mL of chloroform, 400 mL of 80% methanol solution is used as an elution reagent, and the eluent is collected in equal parts until the sample is eluted.

[016] The present invention discloses a method for preparing a spermidine extract from animal muscle tissues by ultrasonication and extraction, rotary evaporation, vacuum freeze-drying and silica gel column chromatography for purification. The present invention provides a simple, convenient, fast, efficient and low-cost extraction

method for extraction of natural spermidine. The method is practical and has the advantages of strong operability, short extraction time and high repeatability.

BRIEF DESCRIPTION OF THE FIGURES

[017] Fig. 1 is a flow chart of extraction, concentration, purification and detection of spermidine.

[018] Fig. 2 shows chromatographic peaks of spermidine (reference standard) and 1,6-hexamethylenediamine (internal standard). The chromatographic peak of spermidine shows that spermidine has good peak separation, and the retention time of spermidine is 6.248 min, the chromatographic peak of the internal standard shows that the internal standard has a good peak, and the retention time of the internal standard is 5.718 min.

[019] Fig. 3 is a standard curve of spermidine reference standard. The linear equation of the spermidine standard curve is $y=0.0047x+0.0201$, $R^2=0.9997$. $R^2>0.99$ indicates that the standard curve has a good linear relationship.

[020] Fig. 4 shows chromatographic peaks of spermidine (spermidine extract) and 1,6-hexamethylenediamine (internal standard). Both spermidine and the internal standard have good peak separation, and the retention time of spermidine and 1,6-hexamethylenediamine is 6.184 min and 5.631 min, respectively.

DESCRIPTION OF THE INVENTION

[021] The present invention will be further described in detail with reference to preferred embodiments, but the scope of the present invention should not be construed as limited to the embodiments set forth herein. Without departing from the spirit and essence of the present invention, all modifications or replacements made to the method, steps or conditions of the present invention fall within the scope of the present invention.

[022] Unless otherwise specified, all biochemical reagents used in the embodiments are commercially available reagents, and the technical means used in the embodiments are conventional means known to a person skilled in the art.

[023] Instruments and reagents

[024] Glass sand core chromatographic column with standard inlet (22 mm × 30 cm); vacuum freeze-dryer; RE3000A rotary evaporator; Agilent 1260 (California) high performance liquid chromatograph; UV detector; chromatographic column SWEEL Chromstar^{TM} C18 (5 μm, 4.6×250 mm); electronic scale up to 0.1 mg; SB-3200DT CNC ultrasonic cleaner: Thermo centrifuge; water bath; Thermo Hypersep C18 SPE column; ultrapure water purifier. Chromatographic methanol; 60% ethanol; chloroform; 60Å, 200-425 mesh silica gel; chromatographic spermidine; chromatographic 1,6-hexamethylenediamine; ultrapure water; NaOH; HCl; analytically pure benzoyl chloride; $HClO_4$.

[025] Ultrasonic extraction

[026] Accurately weighing and putting 1.0 g of porcine muscle tissue in a 15 mL centrifuge tube, and adding 5 mL of 60% ethanol (V/V);

[027] transferring all the muscle tissue and liquid to a glass homogenizer for homogenization until there was no visible particles, then transferring the homogenate to a 50 mL centrifuge tube, eluting the homogenizer with 3 mL of 60% ethanol (V/V) for more than 3 times until there was no visible attachment on the tube wall of the homogenizer, collecting and transferring the eluent to the centrifuge tube;

[028] sealing the centrifuge tube nozzle with a sealing film, and mixing well by vortex oscillation for 30 s, followed by ultrasonication in an ultrasonic disruptor at 40°C for 60 min;

[029] centrifuging in an ultra-speed refrigerated centrifuge at 12,000 r/min at 4°C for 10 min, collecting and putting the supernatant in a 50 mL centrifuge tube.

[030] adding 20 mL of 60% ethanol (V/V) to the precipitate, and mixing well by vortex oscillation for 30 s, followed by ultrasonication in an ultrasonic cell disruptor for 60 min;

[031] centrifuging in an ultra-speed refrigerated centrifuge at 12,000 r/min at 4°C for 10 min, and transferring the supernatant to a 50 mL centrifuge tube to obtain a crude extract of a spermidine extract from the fresh porcine muscle tissue.

[032] Concentration and freeze-drying

[033] Setting the water bath temperature of the rotary evaporator at 45°C, and adding the crude spermidine extract from the muscle tissue to be subject to rotary evaporation to an evaporating flask at one time;

[034] communicating a condenser tube to ensure that the lower end of the condenser tube is communicated with a water inlet and the upper end is communicated with a drain pipe, with the lower end of the condenser tube connected with a waste liquid receiving flask, fixing the opening and screwing the screw tightly;

[035] connecting a vacuum pump to a vacuum nozzle, checking the air tightness of the device, and turning on the vacuum pump while keeping good air tightness; followed by rotary evaporation for 30 min at a rotating speed of the evaporating flask of 200 r/min until the ethanol is completely removed;

[036] collecting and transferring the remaining crude extract in the evaporating flask to a new 50 mL centrifuge tube; freezing the centrifuge tube quickly in liquid nitrogen, sealing the centrifuge tube nozzle with a 0.22 μm cell strainer, and putting the centrifuge tube in a pre-condensed and pre-depressurized freeze-dryer for freeze-drying at -80°C for 24 h, and collecting the yellowish-white powder product at the bottom of the centrifuge tube.

[037] Separation and purification

[038] Wet packing: weighing and putting 40 g of silica gel in a beaker, adding 20 mL of 80% methanol (V/V) solution to prepare a suspension, and putting the suspension in an ultrasonic disruptor for ultrasonication for 10 min to remove bubbles in the silica gel suspension; injecting the silica gel suspension slowly into a silica gel column with a filter element at one time; rinsing the remaining silica gel in the beaker with 80% methanol (V/V) until the silica gel is completely transferred to the silica gel column; then opening the piston at the lower end of the silica gel column to allow the eluent to flow out slowly, and spreading absorbent cotton with a thickness of 1-2 mm on the silica gel surface after the silica gel surface is flush with the eluent level;

[039] loading a sample; adding 3 mL of chloroform to the freeze-dried sample to fully dissolve the freeze-dried powder, sucking the dissolved sample with a 5 mL needle tube, and filtering with a 0.22 μm syringe-driven filter; adding the filtered sample to the silica gel column slowing at one time, and covering the sample with a layer of 5 mm thick silica gel;

[040] eluting, opening the piston at the lower end of the column and keeping the 80% methanol solution at a flow rate of 0.3 mL/min; arranging a separating funnel at the upper end of the silica gel column to ensure that the methanol flow rate is the same as the eluent outflow rate;

[041] collecting for treatment; collecting 40 mL of eluent in a 50 mL centrifuge tube, with 10 tubes of sample collected in total, and mixing the eluents of tubes 6, 7, 8, 9 and 10 for subsequent operation.

[042] Concentration and freeze-drying

[043] Putting the above solution in a rotary evaporator for rotary evaporation at 45°C for 30 min at a rotating speed of the evaporating flask of 200 r/min to allow the ethanol to rapidly volatilize and condense until complete volatilization;

[044] collecting and transferring the remaining eluent in the evaporating flask to a new 50 mL centrifuge tube; freezing the centrifuge tube quickly in liquid nitrogen, sealing the tube nozzle with a 0.22 μm cell strainer, and putting the centrifuge tube in a pre-condensed and pre-depressurized freeze-dryer for freeze-drying at -80°C for 24 h, and collecting the white powder product at the bottom of the centrifuge tube;

[045] using benzoyl chloride for pre-column derivatization of a spermidine extract, and detecting the spermidine content of the spermidine extract by HPLC under the following detection conditions: chromatographic detection conditions are as follows: loaded sample: 20 μL, detection time: 20 min; mobile phase ratio: methanol: ultrapure water = 62:38 (V/V); flow rate: 1.0 mL/min; detection wavelength of UV detector: 229 nm; column temperature: 40°C.

[046] Example 1 Extraction and determination of spermidine from porcine muscle tissues

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[047] Weighing and grinding 1.0 g of muscle sample fully in a glass homogenizer;

[048] adding 20 mL of 60% ethanol to homogenize with the tissue for ultrasonic extraction at 40°C for 60 min; centrifuging at 12,000 r/min for 10 min, and transferring the supernatant to a 50 mL brown test tube;

[049] adding 20 mL of 60% ethanol to the precipitate, mixing well by vortex oscillation, ultrasounding, centrifuging, transferring the supernatant to the same brown test tube and mixing well;

[050] adding the sample to a distilling flask at one time, setting the water bath temperature at 45°C, rotating at 200 r/min before vacuum concentration, and collecting the distillate to a 15 mL centrifuge tube after evaporation for 30 min;

[051] freezing the sample quickly in liquid nitrogen, and putting the frozen sample into a vacuum freeze-dryer for vacuum-drying at -80°C for 24 h to obtain a freeze-dried crude spermidine extract powder;

[052] weighing and packing 40 g of silica gel into a column, adding 2 mL of chloroform to the centrifuge tube to dissolve the freeze-dried powder, filtering with a 0.22 μm syringe-driven filter, adding the filtered sample slowly to a chromatographic column, and covering the sample with a layer of silica gel;

[053] eluting with 80% methanol solution at an elution rate of 0.5 mL/min; collecting the eluent in equal parts, with 40 mL sample in each part; stopping collecting when the sample is completely eluted;

[054] adding the collected sample to a distilling flask at one time, setting the water bath temperature at 45°C, rotating at 200 r/min before vacuum concentration, and collecting the distillate to a 15 mL centrifuge tube after evaporation for 30 min;

[055] freezing the sample quickly in liquid nitrogen, and putting the frozen sample into a vacuum freeze-dryer for vacuum-drying at -80°C for 24 h to obtain a freeze-dried spermidine extract powder;

[056] using benzoyl chloride for pre-column derivatization of a spermidine extract, and detecting the spermidine content of the spermidine extract by HPLC.

[057] Example 2 Extraction and determination of spermidine from a mouse liver tissues

[058] Weighing and grinding 1.0 g of mouse liver tissue sample in a glass homogenizer;

[059] adding 20 mL of 60% ethanol to homogenize with the tissue for ultrasonic extraction at 40°C for 60 min; centrifuging at 12,000 r/min for 10 min, and transferring the supernatant to a 50 mL brown test tube;

[060] adding 20 mL of 60% ethanol to the precipitate, mixing well by vortex oscillation, ultrasounding, centrifuging, transferring the supernatant to the same brown test tube, and mixing well;

[061] adding the sample to a distilling flask at one time, setting the water bath temperature at 45°C, rotating at 200 r/min before vacuum concentration, and collecting the distillate to a 15 mL centrifuge tube after evaporation for 30 min;

[062] freezing the sample quickly in liquid nitrogen, and putting the frozen sample into a vacuum freeze-dryer for vacuum-drying at -80°C for 24 h to obtain a freeze-dried crude spermidine extract powder;

[063] weighing and packing 40 g of silica gel into a column, adding 3 mL of chloroform to the centrifuge tube to dissolve the freeze-dried powder, filtering with a 0.22 μm syringe-driven filter, adding the filtered sample slowly to a chromatographic column, and covering the sample with a layer of silica gel;

[064] eluting with 80% methanol solution at an elution rate of 0.5 mL/min; collecting the eluent in equal parts, with 40 mL sample in each part; stopping collecting when the sample is completely eluted;

[065] adding the collected sample to a distilling flask at one time, setting the water bath temperature at 45°C, rotating at 200 r/min before vacuum concentration, and collecting the distillate to a 15 mL centrifuge tube after evaporation for 30 min;

[066] freezing the sample quickly in liquid nitrogen, and putting the frozen sample into a vacuum freeze-dryer for vacuum-drying at -80°C for 24 h to obtain a freeze-dried spermidine extract powder.

[067] using benzoyl chloride for pre-column derivatization of a spermidine extract, and detecting the spermidine content of the spermidine extract by HPLC.

[068] Although the invention has been described with reference to specific examples, it will be appreciated by those skilled in the art that the invention may be embodied in many other forms, in keeping with the broad principles and the spirit of the invention described herein.

[069] The present invention and the described embodiments specifically include the best method known to the applicant of performing the invention. The present invention and the described preferred embodiments specifically include at least one feature that is industrially applicable

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for ultrasonic extraction of spermidine from an animal muscle tissue, characterized in that ultrasoniation conditions of the method are as follows: with 60% ethanol (V/V) as an extraction solvent, and the ratio of the tissue sample to the extraction solvent of 1:20 (g: mL), ultrasonic disruption is carried out at a frequency of 40 KHz at 40°C for 60 min.

2. A method for extraction, concentration and purification of spermidine from animal muscle tissues, characterized by comprising the following operation steps:

(1) putting animal muscle tissue samples in liquid nitrogen and grinding the sample into a powder, weighing 1 g of the tissue, adding 5 mL of 60% ethanol (V/V) to the tissue to obtain a mixture, and homogenizing the mixture in a glass homogenizer; adding 15 mL of 60% ethanol (V/V) to the homogenate, mixing well by vortex oscillation, then sealing and placing the homogenate in an ultrasonic disruptor for ultrasonication at 40°C for 60 min; centrifuging in an ultra-speed refrigerated centrifuge at 12,000 r/min at 4°C for 10 min, and collecting the supernatant;

(2) adding 20 mL of 60% ethanol (V/V) to the precipitate, mixing well by vortex oscillation for 30 s before ultrasonication and extraction again, and collecting the supernatant to obtain a crude extract of a spermidine extract from the animal muscle tissue, and adding the supernatant to an evaporating flask at one time for rotary evaporation at 45°C for 30 min; collecting the remaining crude extract in the evaporating flask, and freezing the collected crude extract quickly in liquid nitrogen, sealing the centrifuge tube nozzle with a 0.22 μm cell strainer, and putting the centrifuge tube in a pre-condensed and pre-depressurized freeze-dryer for freeze-drying at -80°C for 24 h;

(3) adding 40 g of silica gel to a chromatographic column, dissolving the freeze-dried sample in 2 mL of chloroform, and then eluting with 400 mL of 80% methanol; collecting eluents from tubes 6, 7, 8, 9 and 10, mixing the eluents to obtain a mixture, and putting the mixture in a rotary evaporator for rotary evaporation at 45°C for 30 min; collecting the remaining crude extract in the evaporating flask, freezing the collected crude extract quickly in liquid nitrogen, sealing the centrifuge tube nozzle with a 0.22 μm cell strainer, and putting the centrifuge tube in a pre-condensed and pre-

depressurized freeze-dryer for freeze-drying at -80°C for 24 h to obtain a spermidine extract.

3. The method for ultrasonic extraction of spermidine according to claim 1 characterized in that the extraction reagent used is 60% ethanol (V/V), the ratio of the amount of the muscle tissue sample to the amount of the extraction solvent is 1:20 (g:mL), and the ultrasonication conditions are 40°C, 40 KHz and 60 min.

4. The method for extraction, concentration and purification of spermidine from animal muscle tissues according to any of claim 2, characterized in that the method is practicable with a rotary evaporation temperature of 45°C and an evaporation time of 30 min; and comprising freeze-drying at -80°C for 24 h; and eluting through a silica gel chromatographic column at a rate of 0.3 mL/min, with 80% methanol (V/V) as an eluent.

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Fig. 1

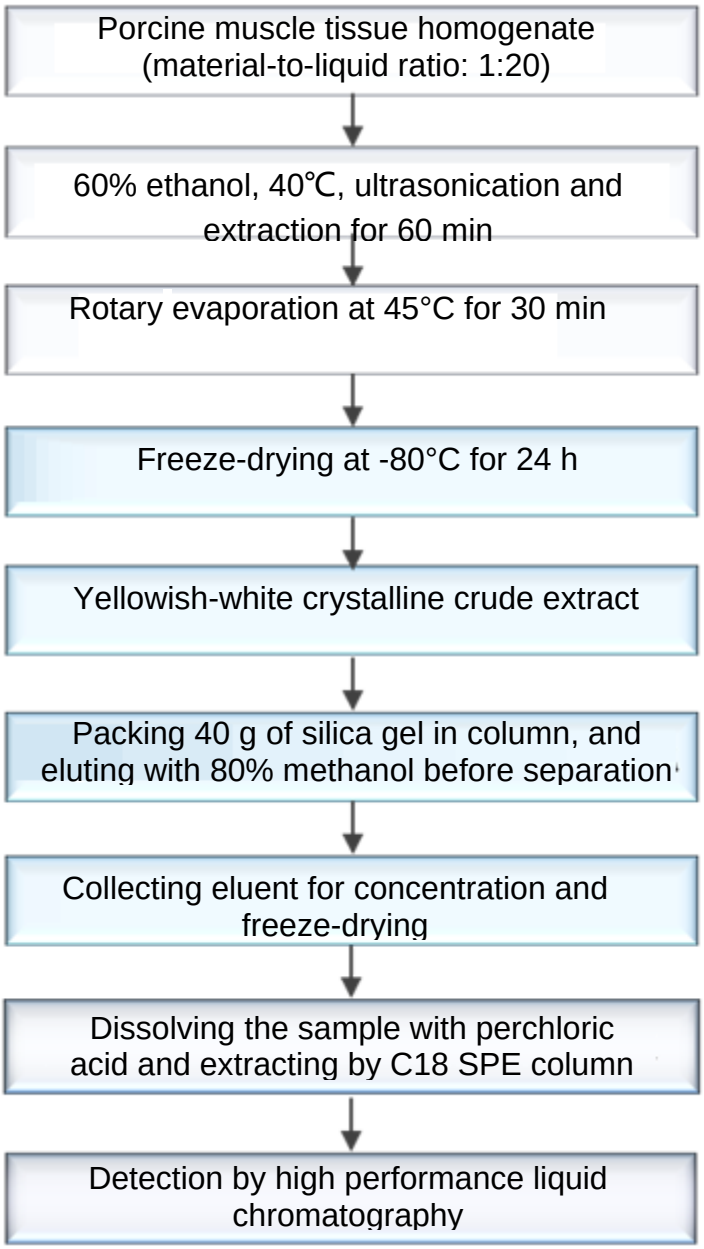

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Fig. 2

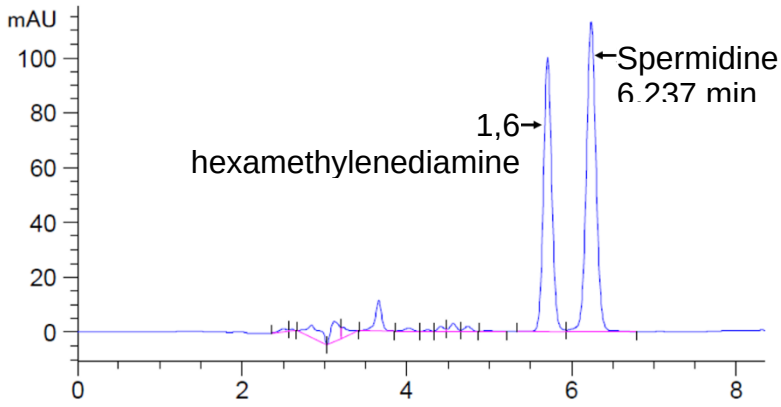

FIG. 3

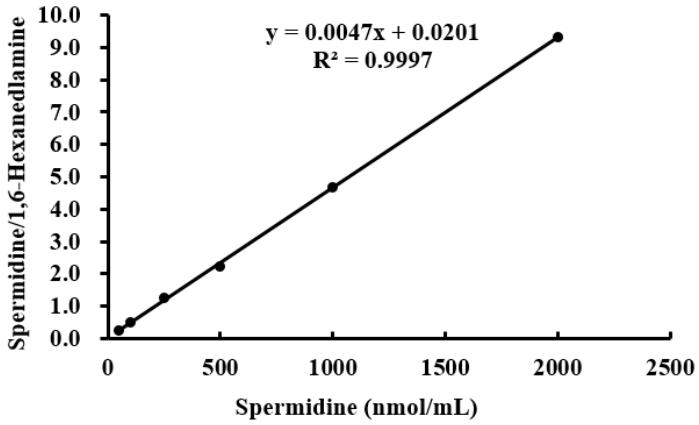

FIG. 4