

METHOD FOR IDENTIFYING GINKGO FORMULA GRANULES AND FRIED GINKGO FORMULA GRANULES BASED ON THIN-LAYER CHROMATOGRAPHY

CROSS REFERENCE TO RELATED APPLICATIONS

5 [0001] This application claims the priority of Chinese Patent Application No. 202311293808.9, filed with the China National Intellectual Property Administration on October 7, 2023, and titled with “METHOD FOR IDENTIFYING GINKGO FORMULA GRANULES AND FRIED GINKGO FORMULA GRANULES BASED ON THIN-LAYER CHROMATOGRAPHY”, which is hereby incorporated by reference in its entirety.

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FIELD

[0002] The present disclosure relates to the technical field of drug detection, specifically relates to a method for identifying Ginkgo Semen formula granules and fried Ginkgo Semen formula granules based on thin-layer chromatography.

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BACKGROUND

[0003] Ginkgo Semen is the dried mature seed of *Ginkgo biloba* L., a plant of the family Ginkgoaceae. It has the efficacies of astringing the lung to relieve asthma, stopping leukorrhea, and reducing urination. However, since the crude Ginkgo Semen is slightly toxic, it needs to be
20 processed before being used as drug in clinical practice. Studies have shown that stir-frying can greatly reduce the toxicity of Ginkgo Semen, thereby improving the accuracy and safety of medication. Moreover, the processed product can enhance the astringent effect and mainly has the efficacies of warming the lung, replenishing qi, relieving cough and asthma, reducing urination, and stopping whitish turbidity urine. Therefore, it can be seen that the chemical components
25 thereof may change after processing.

[0004] Li Zhuanmei et al. compared the contents of ginkgolic acid and total ginkgolic acids in different processed products of Ginkgo Semen, and found that peeling, stir-frying, or steaming and boiling can significantly reduce the contents of ginkgolic acid and total ginkgolic acids in

Ginkgo Semen, thereby reducing toxicity. Among the three methods of stir-frying, steaming, and boiling, the stir-frying method has a higher temperature, a shorter processing time and a good detoxification effect. Li Juan et al. compared the contents of ginkgolide A and ginkgolide C in Ginkgo Semens from different producing regions after stir-frying by using an HPLC-UV method.

5 The results show that, after stir-frying, the contents of ginkgolide A and ginkgolide C in Ginkgo Semens from different producing regions have little difference and are comparable to the amounts lost during processing. Although the contents of ginkgolide A and ginkgolide C in Ginkgo Semen slightly decrease after stir-frying, there is no substantial difference compared with the crude product. Therefore, it is necessary to establish a method with stronger specificity, better
10 repeatability, and effective controllability, so as to provide an effective means for the qualitative identification of fried Ginkgo Semen formula granules.

SUMMARY

[0005] In view of this, the objective of the present disclosure is to provide a method for
15 identifying Ginkgo Semen formula granules and fried Ginkgo Semen formula granules by thin-layer chromatography. The method determines whether a fluorescent spot is present within a certain range of retention factor values in a thin-layer chromatogram, which serves as the basis for identification, thereby identifying Ginkgo Semen formula granules and fried Ginkgo Semen formula granules effectively and rapidly.

20 [0006] To achieve the above objective, the present disclosure adopts the following technical solutions:

[0007] the present disclosure provide a method for identifying Ginkgo Semen formula granules and fried Ginkgo Semen formula granules by thin-layer chromatography, comprising the following steps:

25 [0008] loading a sample solution onto a silica gel thin-layer plate, developing according to thin-layer chromatographic conditions, and after completion of the development, adding a color-developing reagent, heating, and inspecting.

[0009] Preferably, the thin-layer chromatographic conditions are using chloroform - cyclohexane - acetone - glacial acetic acid as a developing solvent, wherein the chloroform,

cyclohexane, acetone, and glacial acetic acid has a volume ratio of (8-12):(3-6):(3-6):(1.5-2.5).

[0010] More preferably, the chloroform, cyclohexane, acetone, and glacial acetic acid have a volume ratio of (10-12) : (5-6) : (5-6) : 2.

[0011] Preferably, loading amount of the sample solution is 15 μ L to 25 μ L.

5 **[0012]** Preferably, the development is performed at an environment temperature of 8°C to 25°C and an environment humidity of 32RH% to 75RH%.

[0013] Preferably, in the inspected thin-layer chromatography, the fried Ginkgo Semen formula granules exhibit a fluorescent spot within ranges of a retention factor R_{f1} of 0.648 ± 0.0648 and a retention factor R_{f2} of 0.741 ± 0.0741 , and the Ginkgo Semen formula granules have no
10 fluorescent spot.

[0014] Preferably, the inspection is performed under ultraviolet light.

[0015] Preferably, the ultraviolet light has a wavelength of 365 nm.

[0016] Preferably, the sample solution is prepared according to the following method:

[0017] mixing a sample with water, heating, filtering, treating the filtrate with column
15 chromatography, eluting with water to remove impurities, eluting with ethanol, collecting the eluate, removing ethanol, extracting with ethyl acetate, evaporating the ethyl acetate phase to dryness, and adding methanol to residue to obtain the sample solution.

[0018] Preferably, the ethanol is 50 vol% ethanol.

[0019] Preferably, the method further comprises a step of loading a reference solution and a
20 negativeneegative solution onto the silica gel thin-layer plate.

[0020] Preferably, reference substances in the reference solution comprise ginkgolide B and ginkgolide C.

[0021] Preferably, loading amount of the reference solution is 1 μ L to 5 μ L;

[0022] Preferably, loading amount of the negative solution is 1 μ L to 3 μ L.

25 **[0023]** Compared with the conventional art, the present disclosure has the following beneficial effects:

[0024] The present disclosure provides a method for identifying Ginkgo Semen formula granules and fried Ginkgo Semen formula granules by thin-layer chromatography. In the method, a sample solution is loaded on a silica gel thin-layer plate and developed under thin-layer chromatographic conditions. The developing solvent used is chloroform - cyclohexane - acetone - glacial acetic acid with a volume ratio of (8-12) : (3-6) : (3-6) : (1.5-2.5). After completion of development, a color-developing reagent is added, followed by heating and inspection. The results show that, in the thin-layer chromatogram, the fried Ginkgo Semen formula granules have fluorescent spots within ranges of a retention factor Rf_1 of 0.648 ± 0.0648 and a retention factor Rf_2 of 0.741 ± 0.0741 , whereas the Ginkgo Semen formula granules have no fluorescent spot. Accordingly, the presence of an additional fluorescent spot within the above Rf_1 range can be used as identification spot 1 for the fried Ginkgo Semen formula granules, and the presence of an additional fluorescent spot within the Rf_2 range can be used as identification spot 2 for the fried Ginkgo Semen kernel formula granules. Verification has shown that the thin-layer chromatography identification method provided in the present disclosure can rapidly and effectively identify fried Ginkgo Semen formula granules which have lost the original decoction piece morphology by identification spots 1 and 2 in the thin-layer chromatogram. The method is simple to operate, has high precision and sensitivity, and exhibits good stability, thereby having good application prospects.

BRIEF DESCRIPTION OF DRAWINGS

[0025] FIG. 1 is a thin-layer chromatogram of a reference solution, a Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution under different loading amounts.

[0026] In FIG. 1, lanes 1-3 correspond to loading amount of the reference solution of 1 μ L, 2 μ L, and 5 μ L, respectively; lanes 4-6 correspond to loading amount of the Ginkgo Semen formula granule solution of 15 μ L, 20 μ L, and 25 μ L, respectively; and lanes 7-9 correspond to loading amounts of the fried Ginkgo Semen formula granule solution of 15 μ L, 20 μ L, and 25 μ L, respectively.

[0027] FIG. 2 is a thin-layer chromatogram of a negative solution, a reference solution, a

Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution.

[0028] In FIG. 2, lane 1 corresponds to the negative solution; lane 2 corresponds to the reference solution; lane 3 corresponds to the Ginkgo Semen formula granule solution; and lane 4 corresponds to the fried Ginkgo Semen formula granule solution.

5 **[0029]** FIG. 3 is a thin-layer chromatogram of a reference solution, a Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution obtained using a silica gel G plate produced by Tianjin Silida Technology Co., Ltd.

[0030] In FIG. 3, lanes 1-3 correspond to the reference solution, the Ginkgo Semen formula granule solution, and the fried Ginkgo Semen formula granule solution, respectively.

10 **[0031]** FIG. 4 is a thin-layer chromatogram of a reference solution, a Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution obtained using a Merck silica gel G plate.

[0032] In FIG. 4, lanes 1-3 correspond to the reference solution, the Ginkgo Semen formula granule solution, and the fried Ginkgo Semen formula granule solution, respectively.

15 **[0033]** FIG. 5 is a thin-layer chromatogram of a reference solution, a Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution obtained using a silica gel G plate produced by Qingdao Ocean Chemical Reagent Factory.

[0034] In FIG. 5, lanes 1-3 correspond to the reference solution, the Ginkgo Semen formula granule solution, and the fried Ginkgo Semen formula granule solution, respectively.

20 **[0035]** FIG. 6 is a thin-layer chromatogram of a reference solution, a Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution developed at a low temperature of 8°C.

[0036] In FIG. 6, lanes 1-3 correspond to the reference solution, the Ginkgo Semen formula granule solution, and the fried Ginkgo Semen formula granule solution, respectively.

25 **[0037]** FIG. 7 is a thin-layer chromatogram of a reference solution, a Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution developed at room temperature of 25°C.

[0038] In FIG. 7, lanes 1-3 correspond to the reference solution, the Ginkgo Semen formula granule solution, and the fried Ginkgo Semen formula granule solution, respectively.

[0039] FIG. 8 is a thin-layer chromatogram of a reference solution, a Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution developed under a humidity environment of 32 RH%.

[0040] In FIG. 8, lanes 1-3 correspond to the reference solution, the Ginkgo Semen formula granule solution, and the fried Ginkgo Semen formula granule solution, respectively.

[0041] FIG. 9 is a thin-layer chromatogram of a reference solution, a Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution developed under a humidity environment of 75 RH%.

[0042] In FIG. 9, lanes 1-3 correspond to the reference solution, the Ginkgo Semen formula granule solution, and the fried Ginkgo Semen formula granule solution, respectively.

[0043] FIG. 10 is a thin-layer chromatogram for verification using a reference solution, multiple batches of Ginkgo Semen formula granule solutions and multiple batches fried Ginkgo Semen formula granule solutions.

[0044] In FIG. 10, lane 1 corresponds to the reference solution; lanes 2-4 correspond to Ginkgo Semen formula granule solutions having batch numbers 2209001, 2209002, and 2209003, respectively; and lanes 5-7 correspond to fried Ginkgo Semen formula granule solutions having batch numbers 2304047, 2304048, and 2304049, respectively.

[0045] FIG. 11 is a thin-layer chromatogram of a reference solution, a Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution obtained using chloroform - cyclohexane - acetone - glacial acetic acid (volume ratio 8:3:3:1.5) as the developing solvent.

[0046] FIG. 12 is a thin-layer chromatogram of a reference solution, a Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution obtained using chloroform - cyclohexane - acetone - glacial acetic acid (volume ratio 12:6:6:2.5) as the developing solvent.

[0047] FIG. 13 is a thin-layer chromatogram of a reference solution, a Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution obtained using the thin-layer chromatographic method of Comparative Example 1.

[0048] In FIG. 13, lane 1 corresponds to a reference solution; lanes 2-3 correspond to a Ginkgo Semen formula granule solution loading at an amount of 10 μ L and 15 μ L, respectively; and lanes 4-5 correspond to a fried Ginkgo Semen formula granule solution loading at an amount of 10 μ L and 15 μ L, respectively.

5 [0049] FIG. 14 is a thin-layer chromatogram of a reference solution, a Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution obtained using chloroform - cyclohexane - acetone - glacial acetic acid (volume ratio 10:10:5:2) as the developing solvent.

[0050] In FIG. 14, lanes 1-2 correspond to the Ginkgo Semen formula granule solution and the fried Ginkgo Semen formula granule solution, respectively.

10 [0051] FIG. 15 is a thin-layer chromatogram of a reference solution, a Ginkgo Semen formula granule solution, and a fried Ginkgo Semen formula granule solution obtained using chloroform - cyclohexane - acetone - glacial acetic acid (volume ratio 5:7:3:1) as the developing solvent.

[0052] In FIG. 15, lane 1 corresponds to the reference solution; lanes 2-3 correspond to the Ginkgo Semen formula granule solution; and lanes 4-5 correspond to the fried Ginkgo Semen
15 formula granule solution.

DETAILED DESCRIPTION

[0053] The technical solutions of the present disclosure will be clearly and completely described below with reference to the embodiments of the present disclosure. Apparently, the
20 described embodiments are only some of the embodiments of the present disclosure rather than all of the embodiments. Based on the embodiments of the present disclosure, all other embodiments obtained by a person of ordinary skill in the art without making creative efforts shall fall within the protection scope of the present disclosure.

[0054] In view of the lack of effective technical methods in the prior art for qualitatively
25 identifying fried Ginkgo Semen formula granules, the present disclosure provides a method for identifying Ginkgo Semen formula granules and fried Ginkgo Semen formula granules by thin-layer chromatography, comprising the following steps:

[0055] loading a sample solution onto a silica gel thin-layer plate, developing according to

thin-layer chromatographic conditions, and after completion of the development, adding a color-developing reagent, heating, and inspecting.

[0056] In the present disclosure, the sample solution refers to a Ginkgo Semen formula granule solution or a fried Ginkgo Semen formula granule solution, which preferably may be prepared according to the following method:

[0057] mixing a sample (i.e., Ginkgo Semen formula granules or fried Ginkgo Semen formula granules) with water, heating, filtering, treating the filtrate with column chromatography, eluting with water to remove impurities, eluting with ethanol, collecting the eluate, removing ethanol, extracting with ethyl acetate, evaporating the ethyl acetate phase to dryness, and adding methanol to residue to obtain the sample solution.

[0058] According to the above method of the present disclosure, an aqueous solution of a sample is first heated to dissolve the sample, followed by filtration to remove insoluble substances. The obtained filtrate containing the sample is purified by column chromatography. The purified sample is then further extracted with ethyl acetate and concentrated to increase the concentration of the sample. Finally, the obtained solid is dissolved in methanol to obtain the corresponding sample solution.

[0059] In the present disclosure, the sample is preferably mixed with water at a ratio of 1 g: (15-25) mL, more preferably 1g : 20 mL, followed by heating to dissolve the sample and filtration to remove insoluble substances. The obtained filtrate containing the sample is then purified by column chromatography. The column chromatography is preferably performed by wet packing, and the column used is a polyamide column. The inner diameter of the polyamide column is preferably 0.8-1.2 cm, more preferably 1 cm. The mesh size of the packed polyamide is preferably 30-60 mesh, more preferably 40-50 mesh, and a mass ratio of the packed polyamide to the sample is preferably 1 : (0.8-1.5), more preferably 1:1. During the column chromatography process, water is preferably used for elution to remove impurities, followed by elution with ethanol, preferably 50 vol% ethanol. After collecting the eluate, the ethanol is preferably removed by evaporation on a water bath. The purified sample is then further extracted with ethyl acetate and concentrated, wherein the extraction is performed twice or more times to increase the concentration of the sample. The ethyl acetate phases are combined and evaporated to dryness,

and the obtained solid is dissolved in methanol. A ratio of the sample to methanol is preferably 1g : (0.8-3)mL, more preferably 1g : 1mL, thereby obtaining the corresponding sample solution.

[0060] In one specific embodiment of the present disclosure, the sample solution is prepared according to the following method:

5 **[0061]** 1 g of sample (i.e., Ginkgo Semen formula granules or fried Ginkgo Semen formula granules) is taken, and 20 mL of water is added. The mixture is heated to dissolve the sample and then filtered. The filtrate is concentrated to 5 mL and loaded onto a pretreated polyamide column (30-60 mesh, 1 g, inner diameter 1 cm, wet-packed with water). The column is eluted with 50 mL of water, and the aqueous eluate is discarded. The column is then eluted with 40 mL of 50 vol%
10 ethanol, and the eluate is collected. The ethanol is evaporated off on a water bath. The remaining aqueous phase is extracted by shaking with ethyl acetate twice, 20 mL each time. The ethyl acetate phases are combined and evaporated to dryness. The residue is dissolved in 1 mL of methanol to obtain the sample solution.

[0062] According to the present disclosure, after the sample solution is obtained, the sample
15 solution is loaded on a silica gel thin-layer plate and developed according to thin-layer chromatographic conditions. After completion of the development, a color-developing reagent is added, followed by heating and inspection. The silica gel thin-layer plate is preferably a silica gel G thin-layer plate. Durability studies of the present disclosure show that silica gel thin-layer plates produced by different manufacturers can achieve the corresponding effects, indicating that
20 the method has good adaptability to different thin-layer plates. Therefore, the source of the silica gel thin-layer plate is not particularly limited. In addition, through investigation of the loading amount, it was found that when the loading amount of the sample solution is 15-25 μ L, the obtained thin-layer chromatogram exhibits clear spots and good separation. Therefore, the loading amount of the sample solution is preferably 15-25 μ L, more preferably 20-25 μ L, and
25 most preferably 25 μ L.

[0063] It should be noted that not all developing solvents can provide a developing effect for Ginkgo Semen formula granules or fried Ginkgo Semen formula granules. Through investigation, the present disclosure preferably selects chloroform - cyclohexane - acetone - glacial acetic acid as the developing solvent in the thin-layer chromatographic conditions. In order to ensure good

separation and clear spot coloration, the present disclosure provides that a volume ratio of chloroform, cyclohexane, acetone, and glacial acetic acid is preferably (8-12):(3-6):(3-6):(1.5-2.5), more preferably (10-12):(5-6):(5-6):2, and most preferably 10:5:5:2.

[0064] In the present disclosure, thin-layer chromatographic separation of the above sample solutions is performed under developing environments with different temperatures and humidities. The results show that the method has strong adaptability to temperature and humidity. The environment temperature for development may be within a range of 8-25°C, specifically may be 8°C, 10°C, 15°C, 20°C, or 25°C and so on. The environment humidity may be within a range of 32-75% RH, specifically may be 32% RH, 35% RH, 40% RH, 45% RH, 50% RH, 55% RH, 60% RH, 65% RH, 70% RH, or 75% RH and so on.

[0065] After completion of the development, in order to display a fluorescent spot during inspection, a color-developing reagent is sprayed onto the silica gel thin-layer plate. The color-developing reagent is preferably an acetic anhydride solution. The plate is heated at 140-160°C, preferably 150°C, for 20-40 minutes, preferably 30 minutes, and then inspected under ultraviolet light (365 nm).

[0066] After inspection, the obtained thin-layer chromatogram of the samples of Ginkgo Semen formula granules or fried Ginkgo Semen formula granules shows that, within a retention factor range of Rf_1 of 0.648 ± 0.0648 (i.e., approximately 0.58-0.71), fried Ginkgo Semen formula granules exhibit one additional fluorescent spot compared with Ginkgo Semen formula granules, which is used as identification spot 1 for fried Ginkgo Semen formula granules. Within a retention factor range of Rf_2 of 0.741 ± 0.0741 (i.e., approximately 0.67-0.82), fried Ginkgo Semen formula granules exhibit one additional fluorescent spot compared with Ginkgo Semen formula granules, which is used as identification spot 2 for fried Ginkgo Semen formula granules. Therefore, the presence of additional fluorescent spots within the above Rf_1 and Rf_2 ranges may be used as identification spots for fried Ginkgo Semen formula granules.

[0067] In some embodiments of the present disclosure, the method further comprises a step of loading a reference solution and a negative solution onto the silica gel thin-layer plate. The reference solution is used to determine the presence of ginkgolide B and ginkgolide C in the sample, and the negative solution is used to exclude interference with the sample.

[0068] In the present disclosure, since the fluorescent spot of ginkgolide A is not clear, after comprehensive consideration, the reference substances in the reference solution preferably include ginkgolide B and ginkgolide C. The reference solution may preferably be prepared by mixing a ginkgolide B reference substance and a ginkgolide C reference substance with methanol.

5 In some specific embodiments of the present disclosure, the reference solution is preferably prepared according to the following steps:

[0069] taking ginkgolide B reference substance and ginkgolide C reference substance, dissolving in methanol to prepare a mixed solution in which each reference substance is present at a concentration of 1 mg per mL, which is used as the reference solution.

10 **[0070]** In the present disclosure, after inspection, the chromatogram of the sample exhibits a fluorescent spot of the same color at positions corresponding to those in the chromatogram of the reference solution, indicating that fried Ginkgo Semen and fried Ginkgo Semen formula granules can be effectively identified.

[0071] The negative solution may be prepared according to the following method:

15 **[0072]** taking 1 mL of methanol as the negative solution.

[0073] It should be noted that, according to investigations of the present disclosure, the loading amount of the reference solution is preferably 1-5 μL , more preferably 2 μL , and the loading amount of the negative solution is preferably 1-3 μL , more preferably 1 μL .

20 **[0074]** Through verification with multiple batches, the thin-layer chromatographic identification method provided in the present disclosure can rapidly and effectively identify fried Ginkgo Semen formula granules which have lost the original decoction piece morphology through the identification spots R_{f1} and R_{f2} in the thin-layer chromatogram. The method is simple to operate, has high precision and sensitivity, and exhibits good stability, thereby having good application prospects.

25 **[0075]** In order to further illustrate the present disclosure, the following embodiments are provided for detailed description. The instruments used in the following embodiments of the present disclosure include a heating plate, a mortar, a semi-automatic thin-layer loading instrument (CAMAG Linomat-5), a thin-layer imaging system (CAMAG TLC Visualizer), and silica gel G thin-layer plates (Qingdao Ocean Chemical Factory, batch No.: 20180527; Tianjin

Silida Technology Co., Ltd., batch No.: 191016; Merck, batch No.: HX87183353). The reagents used include toluene, ethyl acetate, methanol, acetone, and acetic anhydride, all of which are of analytical grade, and water is ultrapure water (prepared in the laboratory). The reagents used include ginkgolide B (National Institutes for Food and Drug Control, batch No.: 110863-201611),
5 ginkgolide C (Sichuan Weikeyi Biotechnology Co., Ltd., batch No.: wkq18051108), Ginkgo Semen formula granules (Sichuan Neo-Green Pharmaceutical Technology Development Co., Ltd., batch Nos.: 2209001, 2209002, 2209003) and fried Ginkgo Semen formula granules (Sichuan Neo-Green Pharmaceutical Technology Development Co., Ltd., batch Nos.: 2304047, 2304048, 2304049).

10 **[0076]** Preparation example 1

[0077] The present preparation example provided a method for preparing a sample solution of Ginkgo Semen formula granules or fried Ginkgo Semen formula granules, comprising the following steps:

15 **[0078]** 1 g of the product (i.e., Ginkgo Semen formula granules or fried Ginkgo Semen formula granules) was taken, and 20 mL of water was added. The mixture was heated to dissolve the sample and then filtered. The filtrate was concentrated to 5 mL and was loaded on a pretreated polyamide column (30-60 mesh, 1 g, inner diameter 1 cm, wet-packed with water). The column was eluted with 50 mL of water, and the aqueous eluate was discarded. The column was then eluted with 40 mL of 50 vol% ethanol, and the eluate was collected. The ethanol was evaporated
20 off on a water bath. The remaining aqueous phase was extracted with ethyl acetate (20 mL each time) twice with shaking. The ethyl acetate phases were combined and evaporated to dryness. The residue was dissolved in 1 mL of methanol to obtain the sample solution.

[0079] Preparation Example 2

25 **[0080]** This preparation example provided a method for preparing a reference solution, comprising the following steps:

[0081] a ginkgolide B reference substance and a ginkgolide C reference substance were taken and dissolved in methanol to prepare a mixed solution containing 1 mg of ginkgolide B reference substance and 1 mg of ginkgolide C reference substance per 1 mL, thereby obtaining a mixed reference solution of ginkgolide B and ginkgolide C (referred to as the “reference solution”).

[0082] Preparation Example 3

[0083] The present preparation example provided a method for preparing a negative solution, comprising the following step:

[0084] 1 mL of methanol was taken as the negative solution.

5 **[0085]** Example 1

[0086] The present example provided a thin-layer chromatographic identification method for Ginkgo Semen formula granules and fried Ginkgo Semen formula granules, and the loading amount was investigated. The method comprised the following steps:

10 **[0087]** S1: The sample solution of Ginkgo Semen formula granules, the sample solution of fried Ginkgo Semen formula granules, and the reference solution were prepared according to the methods described in Preparation Examples 1-2.

[0088] S2: 15 μ L, 20 μ L, and 25 μ L of the sample solution of Ginkgo Semen formula granules and the sample solution of fried Ginkgo Semen formula granules were taken respectively, and 1 μ L, 2 μ L, and 5 μ L of the reference solution were taken. The samples were tested according to the
15 thin-layer chromatography method (General Chapter 0502 of the 2020 Edition of the Chinese Pharmacopoeia). The above solutions were respectively loaded onto the same silica gel G thin-layer plate. Chloroform - cyclohexane - acetone - glacial acetic acid (10:5:5:2, v/v/v/v) was used as the developing solvent. The plate was developed at 25°C and 75 RH%, removed, and air-dried. The plate was sprayed with an acetic anhydride solution and heated at 140°C for 30
20 minutes, and then inspected under ultraviolet light (365 nm).

[0089] The results are shown in FIG. 1, (lanes 1-3 correspond to loading amount of the reference solution of 1 μ L, 2 μ L, and 5 μ L, respectively; lanes 4-6 correspond to loading amount of the Ginkgo Semen formula granule solution of 15 μ L, 20 μ L, and 25 μ L, respectively; and lanes 7-9 correspond to loading amount of the fried Ginkgo Semen formula granule solution of
25 15 μ L, 20 μ L, and 25 μ L, respectively.) It can be seen that when the loading amount of the reference solution was 2 μ L and the loading amount of the sample solution was 20 μ L, the fluorescent spots on the thin-layer chromatogram were clearly visualized. Therefore, the loading amount of the reference solution was determined to be 2 μ L, and the loading amount of the sample solution was determined to be 20 μ L. Furthermore, in comparison with the chromatogram

of the reference solution, obvious fluorescent spots were observed for fried Ginkgo Semen formula granules at a retention factor (R_{f1}) of 0.65 and a retention factor (R_{f2}) of 0.736, whereas no fluorescent spot was observed at the corresponding positions for Ginkgo Semen formula granules. Therefore, the presence or absence of fluorescent spots at a retention factor (R_{f1}) of 0.65 and a retention factor (R_{f2}) of 0.736 may be used as identification spots.

[0090] Example 2:

[0091] The present example provided a thin-layer chromatographic identification method for Ginkgo Semen formula granules and fried Ginkgo Semen formula granules, and the specificity of the method was investigated. The method comprised the following steps:

[0092] S1: The sample solution of Ginkgo Semen formula granules, the sample solution of fried Ginkgo Semen formula granules, the reference solution, and the negative solution were prepared according to the methods described in Preparation Examples 1-3.

[0093] S2: 20 μL of the sample solution of Ginkgo Semen formula granules and 20 μL of the sample solution of fried Ginkgo Semen formula granules were respectively taken, 1 μL of the reference solution was taken, and 1 μL of the negative solution was taken. The test was carried out according to the thin-layer chromatography method (General Chapter 0502 of the 2020 Edition of the Chinese Pharmacopoeia). The above solutions were respectively loaded on the same silica gel G thin-layer plate. Chloroform - cyclohexane - acetone - glacial acetic acid (10:5:5:2, v/v/v/v) was used as the developing solvent. The plate was developed at 25°C and 75 RH%, removed, and air-dried. The plate was sprayed with an acetic anhydride solution and heated at 150°C for 30 minutes, and then inspected under ultraviolet light (365 nm).

[0094] The results are shown in FIG. 2. It can be seen that the negative sample caused no interference with the samples of Ginkgo Semen formula granules and fried Ginkgo Semen formula granules, indicating that the method exhibited good specificity. In comparison with the chromatogram of the reference solution, obvious fluorescent spots were observed for fried Ginkgo Semen formula granules at a retention factor (R_{f1}) of 0.7 and a retention factor (R_{f2}) of 0.771, whereas no fluorescent spot was observed at the corresponding positions for Ginkgo Semen formula granules. Therefore, the presence or absence of fluorescent spots at a retention factor (R_{f1}) of 0.7 and a retention factor (R_{f2}) of 0.771 may be used as identification spots.

[0095] Example 3

[0096] The present example provided a thin-layer chromatographic identification method for Ginkgo Semen formula granules and fried Ginkgo Semen formula granules, and the durability (different thin-layer plates) of the method was investigated. The method comprised the following steps:

[0097] S1: The sample solution of Ginkgo Semen formula granules, the sample solution of fried Ginkgo Semen formula granules, and the reference solution were prepared according to the methods described in Preparation Examples 1-2.

[0098] S2: 20 μ L of the sample solution of Ginkgo Semen formula granules and 20 μ L of the sample solution of fried Ginkgo Semen formula granules were respectively taken, and 1 μ L of the reference solution was taken. The test was carried out according to the thin-layer chromatography method (General Chapter 0502 of the 2020 Edition of the Chinese Pharmacopoeia). The above solutions were respectively loaded on silica gel G plates obtained from Qingdao Ocean Chemical Reagent Factory, Tianjin Silida Technology Co., Ltd., and Merck. Chloroform - cyclohexane - acetone - glacial acetic acid (10:5:5:2, v/v/v/v) was used as the developing solvent. The plates were developed at 25°C and 75 RH%, removed, and air-dried. The plates were sprayed with an acetic anhydride solution and heated at 160°C for 30 minutes, and then inspected under ultraviolet light (365 nm).

[0099] The results are shown in FIGs. 3-5. FIG. 3 shows the thin-layer chromatogram obtained by using the silica gel G plate from Tianjin Silida Technology Co., Ltd.; FIG. 4 shows the thin-layer chromatogram obtained by using the Merck silica gel G plate; and FIG. 5 shows the thin-layer chromatogram obtained by using the silica gel G plate from Qingdao Ocean Chemical Reagent Factory. As shown in FIGs. 3-5, all three silica gel G plates satisfied the identification requirements, indicating that the method exhibited good durability. Furthermore, in comparison with the chromatogram of the reference solution, obvious fluorescent spots were observed for fried Ginkgo Semen formula granules at retention factor (R_{f1}) of 0.7, 0.658, and 0.7, and retention factor (R_{f2}) of 0.8, 0.734, and 0.791, respectively, whereas no fluorescent spot was observed at the corresponding positions for Ginkgo Semen formula granules. Therefore, the presence or absence of fluorescent spots at retention factor (R_{f1}) of 0.7, 0.658, and 0.7, and

retention factor (R_{f2}) of 0.8, 0.734, and 0.791, may be used as identification spots.

[00100] Example 4:

[00101] The present example provided a thin-layer chromatographic identification method for Ginkgo Semen formula granules and fried Ginkgo Semen formula granules, and the durability (different temperature) of the method was investigated. The method comprised the following steps:

[00102] S1: The sample solution of Ginkgo Semen formula granules, the sample solution of fried Ginkgo Semen formula granules, and the reference solution were prepared according to the methods described in Preparation Examples 1-2.

[00103] S2: 20 μ L of the sample solution of Ginkgo Semen formula granules and 20 μ L of the sample solution of fried Ginkgo Semen formula granules were respectively taken, 1 μ L of the reference solution was taken, and 1 μ L of the negative solution was taken. The test was carried out according to the thin-layer chromatography method (General Chapter 0502 of the 2020 Edition of the Chinese Pharmacopoeia). The above solutions were respectively loaded on the same silica gel G thin-layer plate. Chloroform - cyclohexane - acetone - glacial acetic acid (10:5:5:2, v/v/v/v) was used as the developing solvent. The plate was developed at a low temperature of 8°C and at room temperature of 25°C (relative humidity: 75 RH%), respectively. After development, the plate was removed and air-dried, sprayed with an acetic anhydride solution, heated at 145°C for 30 minutes, and inspected under ultraviolet light (365 nm).

[00104] The results are shown in FIGs. 6-7. FIG. 6 shows the thin-layer chromatogram obtained at 8°C, and FIG. 7 shows the thin-layer chromatogram obtained at 25°C. As shown in FIGs. 6-7, the method exhibited good durability with respect to different temperatures. In comparison with the chromatogram of the reference solution, obvious fluorescent spots were observed for fried Ginkgo Semen formula granules at retention factor (R_{f1}) of 0.6, 0.6 and retention factor (R_{f2}) of 0.7, 0.7 under both a low temperature of 8°C and room temperature of 25°C, whereas no fluorescent spot was observed at the corresponding positions for Ginkgo Semen formula granules. Therefore, the presence or absence of fluorescent spots at retention factor (R_{f1}) of 0.6, 0.6 and retention factor (R_{f2}) of 0.7, 0.7 may be used as identification spots.

[00105] Example 5:

[00106] The present example provided a thin-layer chromatographic identification method for Ginkgo Semen formula granules and fried Ginkgo Semen formula granules, and the durability (different humidities) of the method was investigated. The method comprised the following steps:

[00107] S1: The sample solution of Ginkgo Semen formula granules, the sample solution of fried Ginkgo Semen formula granules, and the reference solution were prepared according to the methods described in Preparation Examples 1-2.

[00108] S2: 20 μL of the sample solution of Ginkgo Semen formula granules and 20 μL of the sample solution of fried Ginkgo Semen formula granules were respectively taken, and 1 μL of the reference solution was taken. The test was carried out according to the thin-layer chromatography method (General Chapter 0502 of the 2020 Edition of the Chinese Pharmacopoeia). The above solutions were respectively loaded on the same silica gel G thin-layer plate. Chloroform - cyclohexane - acetone - glacial acetic acid (10:5:5:2, v/v/v/v) was used as the developing solvent. The plate was developed at humidity conditions of 32 RH% and 75 RH%, respectively (temperature: 25°C) respectively. After development, the plate was removed and air-dried, sprayed with an acetic anhydride solution, heated at 145°C for 30 minutes, and inspected under ultraviolet light (365 nm).

[00109] The results are shown in FIGs. 8-9. FIG. 8 shows the thin-layer chromatogram developed and obtained at 32 RH%, and FIG. 9 shows the thin-layer chromatogram developed and obtained at 75 RH%. As shown in FIGs. 8-9, the method exhibited good durability with respect to different humidity conditions. In comparison with the chromatogram of the reference solution, obvious fluorescent spots were observed for fried Ginkgo Semen formula granules at retention factor (R_{f1}) of 0.6, 0.6 and retention factor (R_{f2}) of 0.7, 0.74 under humidity conditions of 32 RH% and 75 RH%, respectively, whereas no fluorescent spot was observed at the corresponding positions for Ginkgo Semen formula granules. Therefore, the presence or absence of fluorescent spots at retention factor (R_{f1}) of 0.6, 0.6 and retention factor (R_{f2}) of 0.7, 0.74 may be used as identification spots.

[00110] Example 6:

[00111] The present example verified the above thin-layer chromatographic identification method and comprised the following steps:

[00112] S1: Reference substances, three batches of Ginkgo Semen formula granules, and three batches of fried Ginkgo Semen formula granules were respectively taken. According to the methods described in Preparation Examples 1-2, the corresponding reference solution, sample solutions of Ginkgo Semen formula granules from different batches, and sample solutions of fried Ginkgo Semen formula granules from different batches were provided.

[00113] S2: 20 μ L of the sample solutions of Ginkgo Semen formula granules from different batches and 20 μ L of the sample solutions of fried Ginkgo Semen formula granules from different batches were respectively taken, and 1 μ L of the reference solution was taken. The test was carried out according to the thin-layer chromatography method (General Chapter 0502 of the 2020 Edition of the Chinese Pharmacopoeia). The above solutions were respectively loaded onto the same silica gel G thin-layer plate. Chloroform - cyclohexane - acetone - glacial acetic acid (10:5:5:2, v/v/v/v) was used as the developing solvent. The plate was developed at 25°C and 75 RH%, removed, and air-dried. The plate was sprayed with an acetic anhydride solution, heated at 145°C for 30 minutes, and inspected under ultraviolet light (365 nm).

[00114] The results are shown in FIG. 10. In comparison with the chromatogram of the reference solution, obvious fluorescent spots were observed on the test chromatogram of different batches for fried Ginkgo Semen formula granules at retention factor (R_{f1}) of 0.673 and retention factor (R_{f2}) of 0.74, whereas no fluorescent spot was observed at the corresponding positions for Ginkgo Semen formula granules. Therefore, the presence or absence of fluorescent spots at retention factor (R_{f1}) of 0.673 and retention factor (R_{f2}) of 0.74 may be used as identification spots.

[00115] Example 7:

[00116] The present example provided a thin-layer chromatographic identification method for Ginkgo Semen formula granules and fried Ginkgo Semen formula granules. Compared with the method described in Example 6, the only difference was that chloroform - cyclohexane - acetone - glacial acetic acid at a volume ratio of 8:3:3:1.5 was used as the developing solvent, while the remaining parameters and steps were the same as those in Example 1.

[00117] The results are shown in FIG. 11. In comparison with the chromatogram of the reference solution, obvious fluorescent spots were observed for fried Ginkgo Semen formula

granules at retention factor (R_{f1}) of 0.65 and retention factor (R_{f2}) of 0.74, whereas no fluorescent spot was observed at the corresponding positions for Ginkgo Semen formula granules. Therefore, the presence or absence of fluorescent spots at retention factor (R_{f1}) of 0.65 and retention factor (R_{f2}) of 0.74 may be used as identification spots.

5 **[00118]** Example 8:

[00119] The present example provided a thin-layer chromatographic identification method for Ginkgo Semen formula granules and fried Ginkgo Semen formula granules. Compared with the method described in Example 6, the only difference was that chloroform - cyclohexane - acetone - glacial acetic acid at a volume ratio of 12:6:6:2.5 was used as the developing solvent, while the
10 remaining parameters and steps were the same as those in Example 1.

[00120] The results are shown in FIG. 12. In comparison with the chromatogram of the reference solution, obvious fluorescent spots were observed for fried Ginkgo Semen formula granules at retention factor (R_{f1}) of 0.65 and retention factor (R_{f2}) of 0.77, whereas no fluorescent spot was observed at the corresponding positions for Ginkgo Semen formula granules.
15 Therefore, the presence or absence of fluorescent spots at retention factor (R_{f1}) of 0.65 and retention factor (R_{f2}) of 0.77 may be used as identification spots.

[00121] Determination of identification spots R_f for Ginkgo Semen formula granules and fried Ginkgo Semen formula granules

[00122] All retention factor data obtained from the thin-layer chromatographic method
20 validation were summarized, and the results are shown in Table 1. According to the summarized results, the retention factor (R_{f1}) of Identification Spot 1 for fried Ginkgo Semen formula granules was determined to be $0.648 \pm 10\%$, with a specified value of 0.648. The retention factor (R_{f2}) of Identification Spot 2 for fried Ginkgo Semen formula granules was determined to be $0.741 \pm 10\%$, with a specified value of 0.741.

25

Table 1

Test	Identification Spot 1				Identification Spot 2			
	retention factor R_{f1}	Average value	RSD %	Range	retention factor R_{f2}	Average value	RSD %	Range
Loading amount	0.65	0.648	6.9	0.58-0.71	0.736	0.741	4.9	0.67-0.82
Specificity	0.700				0.771			
Silica thin-layer plate	0.700				0.800			
Merck thin-layer plate	0.658				0.734			
Qingdao Ocean thin-layer plate	0.700				0.791			
Different temperature -8°C	0.600				0.700			
Different temperature -25°C	0.600				0.700			
Different humidity -32 RH%	0.600				0.700			
Different humidity -75 RH%	0.600				0.740			
Different batches verification	0.673				0.740			

[00123] Comparative Example 1

[00124] S1: 1 g of Ginkgo Semen formula granules and fried Ginkgo Semen formula granules was respectively taken, finely ground, and dissolved in 30 mL of water. The solution was extracted with ethyl acetate twice (20 mL each time) with shaking. The ethyl acetate phases were combined and evaporated to dryness. The residue was dissolved in 1 mL of methanol to obtain the sample solution. A ginkgolide B reference substance and a ginkgolide C reference substance were taken and dissolved in methanol to prepare a mixed solution containing 1 mg of each per 1 mL, which was used as the reference solution.

[00125] S2: The test was carried out according to the thin-layer chromatography method (General Chapter 0502 of the 2020 Edition of the Chinese Pharmacopoeia). 8 μ L of the sample solution and 2 μ L of the reference solution were respectively loaded onto the same silica gel G thin-layer plate using sodium carboxymethyl cellulose solution containing 4% sodium acetate as

the binder. Toluene - ethyl acetate - acetone - methanol (10:5:5:0.6) was used as the developing solvent. The plate was developed at 25°C and 75 RH%, removed, and air-dried. The plate was sprayed with acetic anhydride, heated at 140°C for 30 minutes, and inspected under ultraviolet light (365 nm).

5 **[00126]** The results are shown in FIG. 13. It was observed that when using the above thin-layer chromatographic identification method, no obvious differences were observed between the spots of Ginkgo Semen formula granules and fried Ginkgo Semen formula granules, and therefore the two could not be effectively distinguished.

10 **[00127]** The above results indicate that when a thin-layer chromatographic method other than that provided in the present disclosure was used, the obtained chromatogram could not effectively identify Ginkgo Semen formula granules from fried Ginkgo Semen formula granules.

[00128] Comparative Example 2:

15 **[00129]** S1: The sample solution of Ginkgo Semen formula granules, the sample solution of fried Ginkgo Semen formula granules, and the reference solution were prepared according to the methods described in Preparation Examples 1-2.

20 **[00130]** S2: The test was carried out according to the thin-layer chromatography method (General Chapter 0502 of the 2020 Edition of the Chinese Pharmacopoeia). 8 µL of the sample solution and 2 µL of the reference solution were respectively loaded onto the same silica gel G thin-layer plate. Chloroform - cyclohexane - acetone - glacial acetic acid (10:10:5:2) was used as the developing solvent. The plate was developed at 25°C and 75 RH%, removed, and air-dried. The plate was sprayed with an acetic anhydride solution and heated at 140-160°C for 30 minutes, and then inspected under ultraviolet light (365 nm).

25 **[00131]** The results are shown in FIG. 14. It was observed that when the above thin-layer chromatographic identification method was used, the separation between Ginkgo Semen formula granules and fried Ginkgo Semen formula granules was poor, and therefore the two could not be effectively distinguished.

[00132] The above results indicate that when using a developing solvent other than the ratio that provided in the present disclosure, the obtained thin-layer chromatogram could not effectively identify Ginkgo Semen formula granules from fried Ginkgo Semen formula granules.

[00133] Comparative Example 3:

[00134] S1: The sample solution of Ginkgo Semen formula granules, the sample solution of fried Ginkgo Semen formula granules, and the reference solution were prepared according to the methods described in Preparation Examples 1-2.

5 **[00135]** S2: 20 μ L of the sample solution of Ginkgo Semen formula granules and 20 μ L of the sample solution of fried Ginkgo Semen formula granules were respectively taken, and 1 μ L of the reference solution was taken. The test was carried out according to the thin-layer chromatography method (General Chapter 0502 of the 2020 Edition of the Chinese Pharmacopoeia). The above solutions were respectively loaded onto the same silica gel G thin-layer plate. Chloroform -
10 cyclohexane - acetone - glacial acetic acid (5:7:3:1, v/v/v/v) was used as the developing solvent. The plate was developed at 25°C and 75 RH%, removed, and air-dried. The plate was sprayed with an acetic anhydride solution and heated at 140-160°C for 30 minutes, and then inspected under ultraviolet light (365 nm).

[00136] The results are shown in FIG. 15. It was observed that when the above thin-layer chromatographic identification method was used, the separation between Ginkgo Semen formula granules and fried Ginkgo Semen formula granules was poor, and therefore the two could not be
15 effectively distinguished.

[00137] The above results indicate that when using a developing solvent other than the ratio that provided in the present disclosure, the obtained thin-layer chromatogram could not effectively
20 identify Ginkgo Semen formula granules from fried Ginkgo Semen formula granules.

[00138] The above description of the disclosed embodiments enables those skilled in the art to achieve or use the present disclosure. Various modifications to these embodiments will be apparent to those skilled in the art, and the general principles defined herein may be implemented in other embodiments without departing from the spirit or scope of the present disclosure.
25 Therefore, the present disclosure is not intended to be limited to the embodiments shown herein, but is to be accorded the broadest scope consistent with the principles and novel features disclosed herein.

CLAIMS

1. A method for identifying Ginkgo Semen formula granules and fried Ginkgo Semen formula granules by thin-layer chromatography, comprising steps of:

5 loading a sample solution onto a silica gel thin-layer plate, developing according to thin-layer chromatographic conditions, and after completion of the development, adding a color-developing reagent, heating, and inspecting;

wherein the thin-layer chromatographic conditions are using chloroform - cyclohexane - acetone - glacial acetic acid as a developing solvent, wherein the chloroform, cyclohexane, 10 acetone, and glacial acetic acid has a volume ratio of (8-12):(3-6):(3-6):(1.5-2.5).

2. The method according to claim 1, wherein the chloroform, cyclohexane, acetone, and glacial acetic acid have a volume ratio of (10-12):(5-6):(5-6):2.

15 3. The method according to claim 1, wherein loading amount of the sample solution is 15 μ L to 25 μ L.

4. The method according to claim 1, wherein the development is performed at an environment temperature of 8°C to 25°C and an environment humidity of 32RH% to 75RH%.

20 5. The method according to claim 1, wherein in the inspected thin-layer chromatography, the fried Ginkgo Semen formula granules exhibit a fluorescent spot within ranges of a retention factor Rf_1 of 0.648 ± 0.0648 and a retention factor Rf_2 of 0.741 ± 0.0741 , and the Ginkgo Semen formula granules have no fluorescent spot.

25 6. The method according to claim 1, wherein the inspection is performed under ultraviolet light;

wherein a wavelength of the ultraviolet light is 365 nm.

7. The method according to claim 1, wherein the sample solution is prepared according to the following method:

5 mixing a sample with water, heating, filtering, treating the filtrate with column chromatography, eluting with water to remove impurities, eluting with ethanol, collecting the eluate, removing ethanol, extracting with ethyl acetate, evaporating the ethyl acetate phase to dryness, and adding methanol to residue to obtain the sample solution.

10 8. The method according to claim 7, wherein the ethanol is 50 vol% ethanol.

9. The method according to claim 1, wherein the method further comprises a step of loading a reference solution and a negative solution onto the silica gel thin-layer plate.

15 10. The method according to claim 9, wherein reference substances in the reference solution comprise ginkgolide B and ginkgolide C;

wherein loading amount of the reference solution is 1 μ L to 5 μ L;

wherein loading amount of the negative solution is 1 μ L to 3 μ L.