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National Food Safety Standard

Determination of vitamin A, D, E in food

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Foreword

This standard replaces GB/T 5009.82-2003 “Determination of retinol and tocopherol in food”, GB 5413.9-2010 “National food safety standard - Determination of vitamin A, D, E in foods for infants and young children, milk and milk products”, GB/T 9695.26-2008 “Meat and meat products - Determination of vitamin A content”, GB/T 9695.30-2008 “Meat and meat products - Determination of vitamin E content”, and NY/T 1598-2008 “Determination of tocopherol content in edible vegetable oils - High performance liquid chromatography”.

As compared with GB/T 5009.82-2003, the main changes of this standard are as follows:

- CHANGE the standard name into “National Food Safety Standard Determination of vitamin A, D, E in food";
- ADD the “Determination of vitamin E in food - Normal phase high performance liquid chromatography”;
- ADD the “Determination of vitamin D in food - Liquid chromatography - Tandem mass spectrometry”;
- ADD the “Determination of vitamin D in food - High performance liquid chromatography";
- MODIFY the “Determination of vitamin A and vitamin E in food - Reversed-phase high performance liquid chromatography”;
- MODIFY the reverse phase chromatography separation conditions of vitamin E isomers, which can simultaneously separate and determine 4 tocopherol isomers;
- DELETE the benzopyrene internal standard quantitative method; CHANGE it to using the external standard method for quantitative;
- DELETE the “colorimetry” to determine vitamin A.

National Food Safety Standard

Determination of vitamin A, D, E in food

1 Scope

This standard specifies the determination of vitamin A, vitamin E and vitamin D in food.

The first method of this standard is applicable to the determination of vitamin A and vitamin E in food.

The second method of this standard is applicable to the determination of vitamin E in edible oil, nuts, beans, pepper powder and other foods.

The third method of this standard is applicable to the determination of vitamin D_2 and vitamin D_3 in food.

The fourth method of this standard is applicable to the determination of vitamin D_2 or vitamin D_3 in formulated foods.

Method 1 Determination of vitamin A and vitamin E in food - Reversed-phase high performance liquid chromatography

2 Principles

The vitamin A and vitamin E in the test specimen are saponified (digested by amylase if containing starch), extracted, purified and concentrated, then separated by C_{30} or PFP reversed-phase liquid chromatography column, detected by ultraviolet detector or fluorescence detector, AND quantitative by external standard method.

3 Reagents and materials

Unless otherwise stated, the reagents used in this method are of analytical grade pure AND water is the primary water as specified in GB/T 6682.

3.1 Reagents

3.1.1 Absolute ethanol (C_2H_5OH): it shall not contain aldehydes after inspection, AND the inspection method is as shown in A.1.

3.3.2.3 γ-tocopherol ($C_{28}H_{48}O_2$, CAS No.: 54-28-4): purity ≥ 95%, OR the standard substance as certified by the state and awarded with the standard substance certificate.

3.3.2.4 δ-Tocopherol ($C_{27}H_{46}O_2$, CAS No.: 119-13-1): purity ≥ 95%, OR the standard substance as certified by the state and awarded with the standard substance certificate.

3.4 Standard solution preparation

3.4.1 Vitamin A standard stock solution (0.500 mg/mL): accurately WEIGH 25.0 mg vitamin A standard substance; USE absolute ethanol to dissolve it; TRANSFER it into a 50 mL volumetric flask; MAKE the volume reach to the mark; at this time the solution concentration is about 0.500 mg/mL. TRANSFER the solution into a brown reagent bottle; SEAL it and PRESERVE it at -20 °C in the dark; AND the valid period is 1 month. Before use, INCREASE the temperature of the solution back to 20 °C; and CONDUCT concentration correction (as for the correction method, SEE Appendix B).

3.4.2 Vitamin E standard stock solution (1.00 mg/mL): respectively and accurately WEIGH 50.0 mg of each of α-tocopherol, β-tocopherol γ-tocopherol and δ-Tocopherol; USE absolute ethanol to dissolve it; TRANSFER it into a 50 mL volumetric flask; MAKE the volume reach to the mark; at this time the solution concentration is about 1.00 mg/mL. TRANSFER the solution into a brown reagent bottle; SEAL it and PRESERVE it at -20 °C; AND the valid period is 6 month. Before use, INCREASE the temperature of the solution back to 20 °C; and CONDUCT concentration correction (as for the correction method, SEE Appendix B).

3.4.3 Intermediate solution of vitamin A and vitamin E mixed standard solution: Accurately PIPETTE 1.00 mL of vitamin A standard stock solution and 5.00 mL of vitamin E standard stock solution into one 50 mL volumetric flask; USE methanol make it reach to the mark; at this time, the vitamin A concentration of the solution is 10.0 μg/mL, AND each tocopherol concentration of the vitamin E is 100 μg/mL. PRESERVE it at -20 °C in dark; AND the valid period is half a month.

3.4.4 Vitamin A and vitamin E standard series working solution: respectively and accurately PIPETTE 0.20 mL, 0.50 mL, 1.00 mL, 2.00 mL, 4.00 mL, 6.00 mL of the intermediate solution of vitamin A and vitamin E mixed standard solution into a 10 mL brown volumetric flask; USE methanol to make the volume reach to the mark; the vitamin A concentration of this standard series is 0.20 μg/mL, 0.50 μg/mL, 1.00 μg/mL, 2.00 μg/mL, 4.00 μg/mL, and 6.00 μg/mL, AND the vitamin E concentration is 2.00 μg/mL, 5.00 μg/mL, 10.0 μg/mL, 20.0 μg/mL, 40.0 μg/mL, and 60.0 μg/mL. PREPARE it before use.

Note: saponification period is generally 30 min; after the saponification solution is cooled down, if there is floating oil on the surface, it shall add the appropriate amount of potassium hydroxide solution, AND extend the saponification time appropriately.

5.2.1.2 Starch-containing samples

WEIGH 2 g ~ 5 g (accurate to 0.01 g) of homogenized solid sample OR 50 g (accurate to 0.01 g) of liquid sample into a 150 mL flat-bottomed flask; as for the solid test specimen, it is required to add about 20 mL of warm water; MIX it uniformly; ADD 0.5 g ~ 1 g amylase; PLACE it in the 60 °C water bath for shaking in the dark for 30 min; TAKE it out; ADD 1.0 g of ascorbic acid and 0.1 g of BHT to the enzymatic hydrolysate; MIX it uniformly; ADD 30 mL of absolute ethanol; ADD 10 mL ~ 20 mL potassium hydroxide solution while shaking; after mixing it uniformly, PLACE it in the add 80 °C constant temperature water bath for shaking and saponification for 30 min; after saponification, immediately USE cold water to cool it to room temperature.

5.2.2 Extraction

USE 30 mL of water to transfer the saponification solution into a 250 mL separation funnel; ADD 50 mL of petroleum ether-ether mixture; SHAKE it for extraction for 5 min; TRANSFER the lower solution into another 250 mL separation funnel. ADD 50 mL of petroleum ether-ether mixture and CONDUCT extraction again; COMBINE the ether layers.

Note: If only vitamin A and α-tocopherol are determined, it may use petroleum ether as the extraction agent.

5.2.3 Washing

USE about 100 mL of water to wash the ether layer; REPEAT washing for about 3 times, until washing the ether layer to neutral (it may use the pH test strips to test the pH value of the lower solution); REMOVE the lower aqueous phase.

5.2.4 Concentration

The washed ether layer is filtered through an anhydrous sodium sulfate (about 3 g) into a 250 mL rotary evaporator or nitrogen concentrator. USE about 15 mL of petroleum ether to rinse the separation funnel and anhydrous sodium sulfate for 2 times; INCLUDE it in the evaporation bottle; and CONNECT it to the rotary evaporator or gas concentration instrument; PLACE it in 40 °C water bath for reduced pressure distillation or air concentration; when the ether solution in the bottle is about 2 mL, TAKE the evaporation bottle off; immediately USE nitrogen to blow it to near dry. USE methanol to dissolve the residues in the evaporation bottle and transfer it into a 10 mL volumetric flask; MAKE its volume reach to

f - Conversion factor (vitamin A: f = 1; vitamin E: f = 0.001);

100 - Conversion factor as calculated for the amount of the sample based every 100 grams;

m - The mass of the weighed sample, in grams (g).

The result retains three significant digits.

Note: If the determination results of vitamin E is expressed by the α-tocopherol equivalent (α-TE), it can use the following equation for calculation: Vitamin E (mg α-TE/100 g) = α-tocopherol (mg/100 g) + β-tocopherol (mg/100 g) × 0.5 + γ-tocopherol (mg/100 g) × 0.1 + δ-tocopherol (mg/100 g) x 0.01.

7 Precision

The absolute difference between the two independent determinations obtained under repeatability conditions shall not exceed 10% of the arithmetic mean.

8 Others

When the sampling amount is 5 g AND the constant volume is 10 mL, the UV detection limit of the vitamin A is 10 μg/100 g AND the limit of quantification is 30 μg/100 g; the detection limit of the tocopherol is 40 μg/100 g AND the limit of quantification is 120 μg/100 g.

Method 2 Determination of vitamin E in food - Normal phase high performance liquid chromatography

9 Principles

The vitamin E in the sample was extracted with organic solvent, concentrated, separated by high performance liquid phase chromatography amide column or silica gel column, detected by fluorescence detector, AND quantified by external standard method.

10 Reagents and materials

Unless otherwise stated, the reagents used in this method are of analytical grade pure AND water is the primary water as specified in GB/T 6682.

10.3.3 γ-tocopherol ($C_{28}H_{48}O_2$, CAS No.: 54-28-4): purity ≥ 95%, OR the standard substance as certified by the state and awarded with the standard substance certificate.

10.3.4 δ-Tocopherol ($C_{27}H_{46}O_2$, CAS No.: 119-13-1): purity ≥ 95%, OR the standard substance as certified by the state and awarded with the standard substance certificate.

10.4 Preparation of standard solutions

10.4.1 Vitamin E standard stock solution (1.00 mg/mL): respectively and accurately WEIGH 50.0 mg of each of α-tocopherol, β-tocopherol γ-tocopherol and δ-Tocopherol (accurate to 0.1 mg); USE absolute ethanol to dissolve it in a 50 mL volumetric flask; MAKE the volume reach to the mark; at this time the solution concentration is about 1.00 mg/mL. TRANSFER the solution into a brown reagent bottle; SEAL it and PRESERVE it at -20 °C in the dark; AND the valid period is 6 month. Before use, INCREASE the temperature of the solution back to 20 °C; and CONDUCT concentration correction (as for the correction method, SEE Appendix B).

10.4.2 Intermediate solution of vitamin E standard solution: Accurately PIPETTE 1.00 mL of the vitamin E standard stock solution into one 100 mL volumetric flask; after using nitrogen to purge the ethanol, USE the mobile phase to make its volume reach to the mark; at this time the concentration of each tocopherol is 10.00 μg/mL. SEAL it and PRESERVE it at -20 °C in the dark; AND valid period is half a month.

10.4.3 Vitamin E standard series working solution: respectively and accurately PIPETTE 0.20 mL, 0.50 mL, 1.00 mL, 2.00 mL, 4.00 mL, 6.00 mL of the intermediate solution of vitamin E mixed standard solution into a 10 mL brown volumetric flask; USE mobile phase to make the volume reach to the mark; the concentration of the 4 kinds of tocopherol in this standard series is 0.20 μg/mL, 0.50 μg/mL, 1.00 μg/mL, 2.00 μg/mL, 4.00 μg/mL, and 6.00 μg/mL.

11 Instruments and equipment

11.1 Analytical balance: the sensitivity is 0.1 mg.

11.2 Constant temperature water bath oscillator.

11.3 Rotary evaporator.

11.4 Nitrogen blowing instrument.

11.5 Ultraviolet spectrophotometer.

μm organic filter, PREPARE for the determination by high performance liquid chromatography.

12.2.3 Dry-based plant samples such as nuts, pulses, and chili powder

WEIGH 2 g ~ 5 g of samples (accurate to 0.01 g); USE the Soxhlet extractor or accelerated solvent extractor to extract the vegetable oil; TRANSFER the extraction solvent containing oil into a 250 mL evaporation flask; in the 40 °C water bath, CONDUCT reduced pressure distillation or gas concentration, until it is dried; TAKE off the evaporation bottle; USE 10 mL of mobile phase to transfer the oil into a 25 mL volumetric flask; ADD 0.1 g of BHT; after dissolved through ultrasonic or vortex oscillation, USE mobile phase to make its volume reach to the mark; SHAKE it uniformly. After making the solution pass through the 0.22 μm pore size organic filter, PREPARE for sample injection.

12.3 Chromatographic reference conditions

The chromatographic reference conditions are listed below:

a) Chromatographic column: amide column (column length 150 mm, inner diameter 3.0 mm, particle size 1.7 μm) or equivalent;

b) Column temperature: 30 °C;

c) Mobile phase: n-hexane + [tert-butyl methyl ether-tetrahydrofuran - methanol mixture (20 + 1 + 0.1)] = 90 + 10;

d) Flow rate: 0.8 mL/min;

e) Fluorescence detection wavelength: excitation wavelength is 294 nm AND emission wavelength is 328 nm;

f) Injection volume: 10 μL.

Note: It may use the Si 60 silica gel column (250 mm in column length, 4.6 mm in inner diameter, 5 μm in particle size) to separate the four tocopherol isoforms. It is recommended to use the mobile phase which is mixed by n-hexane and 1, 4-dioxane at the proportion of (95 + 5).

12.4 Production of standard curve

This method uses external standard method for quantitation purposes. Respectively INJECT the vitamin E standard series working solution, from low concentration to high concentration, into the high performance liquid chromatography, in order to determine the corresponding peak area. Using the

14 Precision

The absolute difference between the two independent determinations obtained under repeatability conditions shall not exceed 10% of the arithmetic mean.

15 Others

When the sampling amount is 2 g AND the constant volume is 25 mL, the detection limit of each tocopherol is 50 μg/100 g AND the limit of quantification is 150 μg/100 g.

Method 3 Determination of vitamin D in food - Liquid chromatography - tandem mass spectrometry

16 Principles

After the samples are added with the isotope internal standard of vitamin D_2 and vitamin D_3, it is subjected to potassium hydroxide ethanol solution saponification (the starch-containing sample will be subjected to amylase enzymatic hydrolysis firstly), extraction, purification by silica gel solid phase extraction column, concentration, separation by reversed-phase high performance liquid chromatography C_{18} column, detection by tandem mass spectrometry, AND quantitation by internal standard method.

17 Reagents and materials

Unless otherwise stated, the reagents used in this method are of analytical grade pure AND water is the primary water as specified in GB/T 6682.

17.1 Reagents

17.1.1 Absolute ethanol (C_2H_5OH): Chromatographic purity, it shall not contain aldehydes after inspection, AND the inspection method is as shown in A.1.

17.1.2 Ascorbic acid ($C_6H_8O_6$).

17.1.3 2, 6-di-tert-butyl-p-cresol ($C_{15}H_{24}O$): abbreviated as BHT.

17.1.4 Amylase: activity unit ≥ 100 U/mg.

17.1.5 Potassium hydroxide (KOH).

17.3.4 Vitamin D_3-d_3 internal standard solution ($C_{27}H_{44}O$-d_3): 100 μg/mL.

17.4 Preparation of standard solutions

17.4.1 Vitamin D_2 standard stock solution: accurately WEIGH 10.0 mg of vitamin D_2 standard substance; USE chromatographic purity absolute ethanol to dissolve it and MAKE the volume reach to 100 mL, in order to make its concentration at about 100 μg/mL; TRANSFER it into a brown reagent bottle; SEAL and STORE it in a -20 °C refrigerator, AND the valid period is 3 months. Before use, USE the UV spectrophotometric method to correct its concentration (as for the correction method, SEE Appendix B).

17.4.2 Vitamin D_3 standard stock solution: accurately WEIGH 10.0 mg of vitamin D_2 [translator note: it should be D_3] standard substance; USE chromatographic purity absolute ethanol to dissolve it and MAKE the volume reach to 10 mL, in order to make its concentration at about 100 μg/mL; TRANSFER it into a 100 mL brown reagent bottle; SEAL and STORE it in a -20 °C refrigerator, AND the valid period is 3 months. Before use, USE the UV spectrophotometric method to correct its concentration (as for the correction method, SEE Appendix B).

17.4.3 Vitamin D_2 standard intermediate solution: Accurately ABSORB 10.00 mL of vitamin D_2 standard stock solution; USE the mobile phase to dilute it and MAKE its volume reach to 100 mL; its concentration is about 10.0 μg/mL AND the valid period is 1 month. The exact concentration is converted based on the corrected concentration.

17.4.4 Vitamin D_3 standard intermediate solution: Accurately ABSORB 10.00 mL of vitamin D_3 standard stock solution; USE the mobile phase to dilute it and MAKE its volume reach to 100 mL in a 100 mL brown volumetric flask; its concentration is about 10.0 μg/mL AND the valid period is 1 month. The exact concentration is converted based on the corrected concentration.

17.4.5 Vitamin D_2 and vitamin D_3 mixed standard solution: Accurately ABSORB 10.00 mL of the vitamin D_2 standard intermediate solution and vitamin D_3 standard intermediate solution; USE the mobile phase to dilute it and MAKE its volume reach to 100 mL; its concentration is about 1.00 μg/mL AND the valid period is 1 month.

17.4.6 Vitamin D_2-d_3 and vitamin D_3-d_3 internal standard mixed solution: Respectively PIPETTE 100 μL of vitamin D_2-d_3 standard stock solution of concentration 100 μg/mL and 100 μL of vitamin D_3-d_3 standard stock solution of concentration 100 μg/mL into a 10 mL volumetric flask; USE methanol to make the volume reach to the mark, in order to prepare it to 1 μg/mL mixed internal standard. AND the valid period is 1 month.

Note: The treatment process shall avoid ultraviolet light, AND it shall avoid light as far as possible.

19.2.1 Saponification

19.2.1.1 Starch-free samples

WEIGH 2 g (accurate to 0.01 g) of the homogenized sample into a 50 mL corked centrifuge tube; ADD 100 μL of vitamin D_2-d_3 and vitamin D_3-d_3 mixed internal standard solution AND 0.4 g of ascorbic acid; ADD 6 mL of warm water of about 40 °C; VORTEX it for 1 min; ADD 12 mL of ethanol; VORTEX it for 30 s; then ADD 6 mL of potassium hydroxide solution; after vortex for 30 s, PLACE it in the constant temperature oscillator; in the 80 °C constant temperature water bath in the dark, OSCILLATE it for 30 min (if the sample tissue is relatively dense, it may be taken out every 5 min ~ 10 min for vortex for 0.5 min); TAKE it out and PLACE it in cold water bath to reduce its temperature.

Note: generally the saponification time is 30 min. If after the saponification solution is cooled, there is floating oil at the liquid surface, it is required to add appropriate amount of potassium hydroxide solution, AND appropriately extend the saponification time.

19.2.1.2 Starch-containing samples

WEIGH 2 g (accurate to 0.01 g) of the homogenized sample into a 50 mL corked centrifuge tube; ADD 100 μL of vitamin D_2-d_3 and vitamin D_3-d_3 mixed internal standard solution AND 0.4 g of amylase; ADD 10 mL of warm water of about 40 °C; PLACE it in constant temperature oscillator; after constant temperature oscillation for 30 min at 60 °C in the dark, TAKE it out and PLACE it in the cold water bath to reduce temperature; in the cooled enzymatic hydrolyzate, ADD 0.4 g of ascorbic acid and 12 mL of ethanol; VORTEX it for 30 s; then ADD 6 mL of potassium hydroxide solution; VORTEX it for 30 s; PLACE it in the constant temperature oscillator; MAKE it subjected to saponification for 30 min as in 19.2.1.1.

19.2.2 Extraction

In the cooled saponification solution, ADD 20 mL of n-hexane; MAKE it subjected to vortex extraction for 3 min; CENTRIFUGE it for 3 min at 6000 r/min. TRANSFER the supernatant into a 50 mL centrifuge tube; ADD 25 mL of water; slightly SHAKE it for 30 times; CENTRIFUGE it for 3 min at 6000 r/min; TAKE the upper organic phase and PREPARE for use.

19.2.3 Purification

INJECT the sample of the determined solution sequentially in order to obtain the peak area ratio between the determined sample and the internal standard; based on the standard curve, OBTAIN the concentration of the vitamin D_2 and vitamin D_3 of the determined solution. The response value of the determined sample solution shall be within the linear range of the standard curve; if it is beyond the linear range, it shall reduce the sample size AND treat it in accordance with 19.2 again before sample injection analysis.

20 Presentation of analytical results

The content of vitamin D_2 and vitamin D_3 in the test specimen are calculated in accordance with the equation (3):

$$X = \frac{\rho \times V \times f \times 100}{m} \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (3)$$

Where:

X - The content of vitamin D_2 (or vitamin D_3) in the test specimen, in micrograms per hundred grams (μg/100 g);

ρ - Concentration of vitamin D_2 (or vitamin D_3) of the sample as calculated from the standard curve, in micrograms per milliliter (μg/mL);

V - Constant volume, in milliliters (mL);

f - Dilution factor;

100 - Conversion factor as calculated for the amount of the sample based every 100 grams;

m - The mass of the weighed sample, in grams (g).

The result retains three significant digits.

Note: If the sample contains both vitamin D_2 and vitamin D_3, the determination result of vitamin D is calculated as the sum of the content of vitamin D_2 and vitamin D_3.

21 Precision

The absolute difference between the two independent determinations obtained under repeatability conditions shall not exceed 15% of the arithmetic mean.

months. Before use, USE the UV spectrophotometric method to correct its concentration (as for the correction method, SEE Appendix B).

24.4.3 Vitamin D_2 standard intermediate solution: Accurately ABSORB 10.00 mL of vitamin D_2 standard stock solution; USE the mobile phase to dilute it and MAKE its volume reach to 100 mL; its concentration is about 10.0 μg/mL AND the valid period is 1 month. The exact concentration is converted based on the corrected concentration.

24.4.4 Vitamin D_3 standard intermediate solution: Accurately ABSORB 10.00 mL of vitamin D_3 standard stock solution; USE the mobile phase to dilute it and MAKE its volume reach to the mark in a 100 mL brown volumetric flask; its concentration is about 10.0 μg/mL AND the valid period is 3 month. The exact concentration is converted based on the corrected concentration.

24.4.5 Vitamin D_2 standard use solution: Accurately ABSORB 10.00 mL of Vitamin D_2 standard intermediate solution; USE the mobile phase to dilute it and MAKE its volume reach to the mark in a 100 mL brown volumetric flask; its concentration is about 1.00 μg/mL. The exact concentration is converted based on the corrected concentration.

24.4.6 Vitamin D_3 standard use solution: Accurately ABSORB 10.00 mL of Vitamin D_3 standard intermediate solution; USE the mobile phase to dilute it and MAKE its volume reach to the mark in a 100 mL brown volumetric flask; its concentration is about 1.00 μg/mL. The exact concentration is converted based on the corrected concentration.

24.4.7 Preparation of the standard series solutions

24.4.7.1 When using the vitamin D_2 as internal standard for the determination of vitamin D_3, respectively and accurately PIPETTE 0.50 mL, 1.00 mL, 2.00 mL, 4.00 mL, 6.00 mL, and 10.00 mL of vitamin D_3 standard immediate solution into a 100 mL brown volumetric flask; respectively ADD 5.00 mL of vitamin D_2 internal standard solution; USE methanol to make its volume reach to the mark and MIX it uniformly. The concentration of this standard series working solution is respectively 0.05 μg/mL, 0.10 μg/mL, 0.20 μg/mL, 0.40 μg/mL, 0.60 μg/mL, and 1.00 μg/mL.

24.4.7.2 When using the vitamin D_3 as internal standard for the determination of vitamin D_2, respectively and accurately PIPETTE 0.50 mL, 1.00 mL, 2.00 mL, 4.00 mL, 6.00 mL, and 10.00 mL of vitamin D_2 standard immediate solution into a 100 mL brown volumetric flask; respectively ADD 5.00 mL of vitamin D_3 internal standard solution; USE methanol to make its volume reach to the mark and MIX it uniformly. The concentration of this standard series working solution

26.2.1 Starch-free samples

WEIGH 5 g ~ 10 g (accurate to 0.01 g) of homogenized solid sample OR 50 g (accurate to 0.01 g) of liquid sample into a 150 mL flat-bottomed flask; ADD 20 mL ~ 30 mL of warm water to the solid sample; ADD 1.00 mL of internal standard use solution (if determining vitamin D_2, USE the vitamin D_3 as the internal standard; if determining vitamin D_3, USE the vitamin D_2 as the internal standard), then ADD 1.0 g of ascorbic acid and 0.1 g of BHT; MIX it uniformly. ADD 30 mL of absolute ethanol; ADD 10 mL ~ 20 mL of potassium hydroxide solution while shaking it; after mixing it uniformly, MAKE it subjected to 80 °C backflow saponification for 30 min in the constant magnetic stirrer; after saponification, immediately USE cold water to cool it down to room temperature.

Note: generally the saponification time is 30 min. If after the saponification solution is cooled, there is floating oil at the liquid surface, it is required to add appropriate amount of potassium hydroxide solution, AND appropriately extend the saponification time.

26.2.2 Starch-containing samples

WEIGH 5 g ~ 10 g (accurate to 0.01 g) of homogenized solid sample OR 50 g (accurate to 0.01 g) of liquid sample into a 150 mL flat-bottomed flask; ADD 20 mL of warm water to the solid sample; ADD 1.00 mL of internal standard use solution (if determining vitamin D_2, USE the vitamin D_3 as the internal standard; if determining vitamin D_3, USE the vitamin D_2 as the internal standard) and 1 g of amylase; PLACE it in the 60 °C constant temperature water batch for oscillation for 30 min; ADD 1.0 g of ascorbic acid and 0.1 g of BHT into the enzymatic hydrolysate; MIX it uniformly. ADD 30 mL of absolute ethanol; ADD 10 mL ~ 20 mL of potassium hydroxide solution while shaking it; after mixing it uniformly, MAKE it subjected to 80 °C backflow saponification for 30 min in the constant magnetic stirrer; after saponification, immediately USE cold water to cool it down to room temperature.

26.2.3 Extraction

USE 30 mL of water to transfer the saponification solution into a 250 mL separation funnel; ADD 50 mL of petroleum ether; MAKE it subjected to oscillation and extraction for 5 min; TRANSFER the lower solution into another 250 mL separation funnel; ADD 50 mL of petroleum ether for re-extraction; COMBINE the ether layer.

26.2.4 Washing

TAKE about 1.00 mL of vitamin D_2 and vitamin D_3 standard intermediate solution into a 10 mL stoppered test tube; at the 40 °C ± 2 °C nitrogen blowing instrument, BLOW it dry. USE 10 mL of n-hexane to oscillate and dissolve it. TAKE 100 μL of this solution and INJECT it into the liquid chromatograph for determination, in order to determine the retention time of vitamin D. Then INJECT the 500 μL determined solution into a liquid chromatograph; based on the retention time of the vitamin D standard solution, COLLECT the vitamin D fraction in a test tube. PLACE the test tube into 40 °C water bath; USE nitrogen to blow it dry; TAKE it out and accurately ADD 1.0 mL of methanol; OSCILLATE the residues to dissolve it, which is the vitamin D determined solution.

26.3.2 Reference conditions for reversed-phase liquid chromatography

Reversed-phase liquid chromatography reference conditions are listed as below:

a) Column: C_{18} column, column length 250 mm, column inner diameter 4.6 mm, particle size 5 μm, or the chromatography column of equivalent performance;

b) Mobile phase: methanol + water = 95 + 5;

c) Flow rate: 1 mL/min;

d) Detection wavelength: 264 nm;

e) Column temperature: 35 °C ± 1 °C;

f) Injection volume: 100 μL.

26.4 Production of standard curve

Respectively INJECT the sample of vitamin D_2 or vitamin D_3 standard series working solution into the reversed-phase liquid chromatography, in order to obtain the peak area of the vitamin D_2 and vitamin D_3; USE the peak area as the ordinate, AND the concentration of vitamin D_2 and vitamin D_3 standard series working solution as the abscissa; respectively DRAW the vitamin D_2 and vitamin D_3 standard curve.

26.5 Determination of sample

PIPETTE 100 μL of the vitamin D determined solution into the reversed-phase liquid chromatograph, in order to obtain the peak area ratio between the determined solution and the internal standard substance; based on the standard curve, OBTAIN the concentration of vitamin D_2 (or vitamin D_3) in the determined solution.

Appendix A

A.1 Examination methods of aldehydes in absolute ethanol

A.1.1 Reagents

A.1.1.1 Silver nitrate.

A.1.1.2 Sodium hydroxide.

A.1.1.3 Ammonia water.

A.1.2 Reagent preparation

A.1.2.1 5% silver nitrate solution: WEIGH 5.00 g of silver nitrate; ADD 100 mL of water to dissolve it; STORE it in a brown reagent bottle.

A.1.2.2 10% sodium hydroxide solution: WEIGH 10.00g sodium hydroxide; ADD 100 mL of water to dissolve it; STORE it in a polyethylene bottle.

A.1.2.3 Silver ammonia solution: ADD ammonia water into 5% silver nitrate, until the resulting precipitate is re-dissolved. ADD a few drops of 10% sodium hydroxide solution. If precipitation occurred, ADD ammonia water again until the precipitate is dissolved.

A.1.3 Operation method

PIPETTE 2 mL of silver ammonia solution into the test tube; ADD a small amount of ethanol; SHAKE it uniformly; then ADD sodium hydroxide solution; HEAT it; PLACE to let it cool down; if there is silver mirror reaction, it indicates that there is aldehydes in the ethanol.

A.1.4 Results treatment

REPLACE with the chromatographic purity absolute ethanol or CONDUCT de-aldehyde treatment for the current ethanol: TAKE 2 g of silver nitrate and DISSOLVE it in a small amount of water; TAKE 4 g of sodium hydroxide and DISSOLVE it in warm ethanol; POUR the both into 1 L of ethanol; after shaking it, PLACE it in the dark for 2 d, during which it shall be shaken from time to time; after filtration, PLACE it in the distillation bottle for distillation; DISCARD the 150 mL initial distillate.

A.2 Examination method of peroxide in ether

A.2.1 Reagents

Appendix B

Vitamin A, D, E standard solution concentration calibration method

After the Vitamin A, vitamin D, vitamin E standard solution is prepared, it shall correct its concentration before use, with the specific operation as follows:

a) TAKE 50 μL of the retinol standard solution into a 10 mL brown volumetric flask; USE absolute ethanol to make its volume reach to the mark; MIX it uniformly; USE 1 cm quartz cuvette determine the absorbance using the absolute ethanol as a blank reference based on the determined wavelength in Table B.1;

b) Respectively PIPETTE 100 μL of the vitamin D_2 and vitamin D_3 standard stock solution into each 10 mL brown volumetric flask; USE absolute ethanol to make its volume reach to the mark; MIX it uniformly; respectively USE 1 cm quartz cuvette determine the absorbance using the absolute ethanol as a blank reference based on the determined wavelength in Table B.1;

c) Respectively PIPETTE 500 μL of α-tocopherol, β-tocopherol, γ-tocopherol and δ-tocopherol standard stock solution into each 10 mL brown volumetric flask; USE absolute ethanol to make its volume reach to the mark; MIX it uniformly; respectively USE 1 cm quartz cuvette determine the absorbance using the absolute ethanol as a blank reference based on the determined wavelength in Table B.1.

The concentration of vitamin A or vitamin E or vitamin D in the determined solution is calculated in accordance with the equation (B.1):

$$X = \frac{A \times 10^4}{E} \qquad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (B.1)$$

Where:

X - Concentration of the vitamin standard diluent, in micrograms per milliliter (μg/mL);

A - The average UV absorbance of the vitamin diluent;

10^4 - Conversion factor;

E - Vitamin 1% colorimetric light coefficient (as for the corresponding colorimetric absorption coefficient of each vitamin, SEE Table B.1).

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