NATIONAL STANDARD FOR FOOD SAFETY OF THE PEOPLE'S REPUBLIC OF CHINA

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

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Foreword

This present National Standard replaces GB/T 5413.9 -1997, *Milk powder and formula foods for infant and young children — Determination of Vitamin A, D, E content.*

This present National Standard, compared with GB/T 5413.9 -1997, has been modified as follows:

— The antioxidant reagent has been modified from original pyrogallic acid to ascorbic acid.

— The heating and refluxing in the pretreatment method has been modified as constant temperature saponification.

— The correction of standard solution has been supplemented.

— The original single point quantification has been modified as the standard curve method.

— The determination of Vitamin D recovery has been supplemented.

— The calculation formula has been modified.

Annex A of this present National Standard is normative, and Annex B is informative.

The original editions replaced by this present National Standard include:

— GB 5413 -1985 and GB/T 5413.9 -1997.

National food safety standard

Determination of vitamin A, D, E in foods for infants and young children,milk and milk products

1. Scope

This present National Standard specifies the method for determination of Vitamin A, D, E in foods for infant and young children, milk and milk products.

This present National Standard is applicable to the determination of vitamin A, D, E content in foods for infant and young children, milk and milk products.

2. Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this present standard. As for the dated references, all the amendments or revisions after them except the corrigenda are not applicable to this present standard. As for the references that are not dated, their most recent editions are applicable to this present national standard.

3. Principle

After saponification and extraction with petroleum ether, Vitamin A and E is separated by reverse phase chromatography method, of which the content is determined by external standard method; Vitamin D is purified by normal phase chromatography method and separated by reverse phase chromatography method, of which the content is determined by external standard method.

4. Reagents and materials

Unless otherwise specified, all reagents shall be analytical pure or higher graded, and all water used for the experiment shall be Grade 1 water specified in GB/T 6682.

4.1 α-Diastase: Enzyme activity ≥ 1.5 U/mg.

4.2 Anhydrous sodium sulfate.

4.3 Isopropyl alcohol: chromatographically pure.

4.4 Alcohol: chromatographically pure.

4.5 Potassium hydroxide solution: Weigh 250 g of solid potassium hydroxide, and dissolve it with 200 mL of water.

4.6 Petroleum ether: boiling range is 30～60℃.

4.7 Methanol: chromatographically pure.

4.8 Normal hexane: chromatographically pure.

4.9 Cyclohexane: chromatographically pure.

4.10 Ethyl alcohol solution of vitamin C: 15 g/L.

4.11 Vitamins A, D, and E standard solutions

4.11.1 Vitamin A standard stock solution (based on retinol) （100 μg/mL）:Weigh 10 mg of standard substance of Vitamin A accurately, dissolve it with ethyl alcohol (4.4) in a 100 mL brown volumetric flask, and dilute to volume.

4.11.2 Vitamin E standard stock solution (based on α- tocopherol)（500 μg/mL）:Weigh 50 mg of standard substance of Vitamin E, dissolve it with ethyl alcohol (4.4) in a 100 mL brown volumetric flask, and dilute to volume.

4.11.3 Vitamin D_2 standard stock solution（100 μg/mL）: Weigh 10 mg of standard substance of Vitamin D_2, dissolve it with ethyl alcohol (4.4) in a 100 mL brown volumetric flask, and dilute to volume.

4.11.4 Vitamin D_3 standard stock solution（100 μg/mL）:Weigh 10 mg of standard substance of Vitamin D_3, dissolve it with ethyl alcohol (4.4) in a 100 mL brown volumetric flask, and dilute to volume.

Note: Vitamin A, D, E standard stock solution should be preserved at the temperature below -10 ℃ away from light. Standard working solution prepared before use. Standard stock solution should be calibrated before use, see Appendix A.

5. Apparatus

5.1 High Performance Liquid Chromatograph, with a UV detector.

5.2 Rotatory evaporator.

5.3 Constant temperature magnetic stirrer.: 20 ℃～80 ℃

5.4 Nitrogen evaporator.

5.5 Centrifuge: rotary speed ≥5000 r/min.

5.6 incubator: 60 □±2 □

5.7 Balance: 0.1 mg of accuracy.

6. Analytical Steps

6.1 Sample pretreatment

6.1.1 Sample containing starch

Weigh about 5 g of solid sample that has been mixed well or about 50 g of liquid sample (accurate to 0.1 mg), and put it into a 250 mL Erlenmeyer flask. Add 1g of α-amylase (4.1). For solid sample, about 50 mL of distilled water at 45～50℃ should be added in to dissolve it. After it is well mixed, use nitrogen gas to exhaust air from the flask. Insert the stopper. Place it in a 60℃±2℃ incubator（5.6）for 30 min.

6.1.2 Sample free of starch

Weigh about 10 g of solid sample that has been mixed well or about 50 g of liquid sample (accurate to 0.1 mg), and put it into a 250 mL Erlenmeyer flask. For solid sample, about 50 mL of distilled water at 45℃～50℃ should be added in to dissolve it, mix well.

6.2 Recovery test should be carried out simultaneously for test of sample with Vitamin D.

6.3 Preparation of test solution

6.3.1 Saponification: Add about 100 mL of ethyl alcohol solution of Vitamin C (4.10) in the above-mentioned sample solution that has been pretreated. After it is well mixed, add 25 mL of the potassium hydroxide solution (4.5). Well mix. Then add magnetic rod and fill nitrogen gas for protection. Insert the rubber stopper. Add about 300 mL water in a 1000 mL of beaker, and place the beaker in a constant temperature magnetic stirrer(5.3) ; when the temperature is controlled at 53 ℃ ±2 ℃, place the triangular flask in a beaker, after saponification under magnetic force stirring for about 45 min, immediately take it out and cool it down to room temperature.

6.3.2 Extraction: Transfer all the saponified solution into a 500 mL separating funnel. Add 100 mL of petroleum ether (4.6) in. Shake it gently. Insert the stopper after exhaust. Severely vibrate it for about 10 min; let stand for demixing. Place the aqueous phase in another 500 mL separating funnel. Repeat the above extraction process. Merge the ether solution, and wash it with distilled water until it becomes neutral. Use anhydrous sodium sulfate for filter and dehydration. Collect the filtrate in a 500 mL flat-bottomed flask. After it is evaporated under 40 ℃±2 ℃ nitrogen gas flow on rotator evaporator and becomes nearly dry (it is not allowed to make it completely dry), use petroleum ether to transfer it into 10 mL volumetric flask, and dilute it to volume.

6.3.3 Take 2.0 mL of petroleum ether solution from the aforesaid volumetric flask and put it into cuvette A. Then take 7.0 mL of petroleum ether solution and put it into cuvette B.

Blow dry the petroleum ether in both cuvette A and B with nitrogen gas respectively in a 40 ℃±2 ℃ of nitrogen evaporator （5.4）. Add 5.0 mL of methanol (4.7) in cuvette A to stir and dissolve the residues. Add 2.0 mL of normal hexane (4.8) in cuvette B to stir and dissolve the residues. Then Centrifuge cuvettes A and B at 5000 r/min for 10 min; take out, let stand until it cools to room temperature before determination. The cuvette A is used for determination of Vitamins A and E, and the cuvette B is used for determination of Vitamin D.

6.4 Determination

6.4.1 Determination of vitamin A and E

6.4.1.1 Reference condition for chromatography:

Chromatographic column: C_{18} column, 250 mm × 4.6 mm, 5 μ, or chromatographic column with the same performance.

Mobile phase: methanol (4.7).

Flow rate: 1.0 mL/min.

Wavelength: Vitamin A, 325 nm; Vitamin E, 294 nm.

Column temperature: 35 ℃±1 ℃

Sample size: 20 μL

6.4.1.2 Drawing of standard curve of Vitamins A and E

Draw 0.50 mL, 1.00 mL, 1.50 mL, 2.00 mL, and 2.50 mL of the Vitamin A standard stock solution (4.11.1) in 50 mL brown volumetric flasks respectively, and dilute them with ethanol to volume; mix well. The concentrations of this series of standard working solutions are 1.00μg/mL, 2.00μg/mL, 3.00μg/mL, 4.00μg/mL, and 5.00 μg/mL respectively.

Draw 1.0 mL, 2.0 mL, 3.0 mL, 4.0 mL and 5.0 mL of the Vitamin E standard stock solution (4.11.2) in 50 mL brown volumetric flasks respectively, and dilute them with ethanol to volume; mix well. The concentrations of this series of standard working solutions are 10.0μg/mL, 20.0μg/mL, 30.0μg/mL, 40.0μg/mL, and 50.0μg/mL respectively.

Inject the standard working solutions of Vitamins A and E into the liquid chromatograph (the chromatograph map sees Annex B) to get the peak heights (or peak areas). Draw the standard curves of Vitamins A and E respectively, in which the y-axis is peak height (or peak area), and the x-axis is the concentration of standard working solutions of Vitamins A and E.

6.4.1.3 Determination of test sample of Vitamins A and E

Inject the test solution (cuvette A in 6.3.3) into the liquid chromatograph to get the peak height (or peak area); consult corresponding standard curve to get the concentrations of Vitamins A and E in test sample solution.

6.4.2 Determination of Vitamin D

6.4.2.1 Purification of test solution of Vitamin D

6.4.2.1.1 Reference condition for chromatography:

Chromatographic column: silica gel column, 150 mm × 4.6 mm, or chromatographic column with the same performance.

Mobile phase: After mixing of cyclohexane (4.9) with normal hexane (4.8) at a volume ratio of 1:1, add 0.8% of isopropyl alcohol (volume ratio) (4.3).

Flow rate: 1 mL/min.

Wavelength: 264 nm.

Column temperature: 35 ℃±1℃.

Injection volume: 500 μL.

6.4.2.1.2 Pipette about 0.5 mL of vitamin D_3 standard stock solution （4.11.4） to a 10mL of test tube with a stopper, and dried at a nitrogen evaporator at 40 ℃±2 ℃（5.4）, the residues are dissolved with 5 mL normal hexane solution by stirring. Inject 50 μL of the Vitamin D standard solution that has been dissolved with normal hexane into liquid chromatograph; determine the retention time of Vitamin D. Then inject 500μL of the sample solution (cuvette B in 6.3.3) into the liquid chromatograph, and collect vitamin D in cuvette C according to the retention time of vitamin D standard solution. Blow dry cuvette C with nitrogen gas at 40 ℃±2℃. Accurately add 1.0 mL of methanol (4.7) to dissolve it; this is the test solution of Vitamin D.

6.4.2.2 Determination of Vitamin D test solution:

6.4.2.2.1 Reference condition for chromatography:

Chromatographic column: C_{18} column, 250 mm × 4.6 mm, 5 μm, or chromatographic column with same performance.

Mobile phase: Methanol (4.7).

Flow rate: 1 mL/min.

Detection wavelength: 264 nm.

Column temperature: 35 ℃ ±1 ℃

Injection volume: 100 μL.

6.4.2.2.2 Drawing of standard curve:

Draw 0.20 mL, 0.40 mL, 0.60 mL, 0.80 mL, and 1.00 mL of the standard stock solution of Vitamin D_2 or D_3 (4.11.3 or 4.11.4) into 100 mL brown volumetric flasks respectively, dilute to volume with ethanol, and mix well. The concentrations of this series of standard working solutions are 0.200μg/mL, 0.400μg/mL, 0.600μg/mL, 0.800μg/mL, and 1.000μg/mL respectively.

Inject the standard working solutions of Vitamin D_2 or D_3 into the liquid chromatograph to get the peak height (or peak area) (see annex B). Draw the standard curve, in which the y-axis is peak height (or peak area), and the x-axis is the concentration of standard working solutions of Vitamin D_2 or D_3.

6.4.2.2.3 Determination of Vitamin D test sample:

Inject 100 μL of test solution of Vitamin D (cuvette C in 6.4.2.1) into the liquid chromatograph (the chromatograph map sees Annex B) to get the peak height (or peak area); consult the standard curve to get the concentrations of Vitamin D_2 (or D_3) in the test sample solution.

The result of recovery test for Vitamin D is recorded as recovery coefficient k, which is then substituted in the calculation formula (Formula (2)) to correct the determination result of Vitamin D content.

7. Expression of analysis results

7.1 Calculation of Vitamin A: The content of Vitamin A in test sample, should be calculated as formula (1):

$$X = \frac{c_s \times 10/2 \times 5 \times 100}{m} \quad \text{..... (1)}$$

In which,

X - the content of vitamin A in the test sample, of which the unit isμg/100 g;

C - the concentration of vitamin A test solution resulted from the standard curve, of which the unit is μg/mL;

m- the mass of sample, of which the unit is gram (g);

Note: 1ug retinol= 3.33 IU vitamin A.

The calculation result of Vitamin A should be expressed as the mean value of two indepdent tests and accurate to three decimal places.

7.2 Calculation of Vitamin D: The content of Vitamin D should be calculated as formula (2):

$$X = \frac{c_s \times 10/7 \times 2 \times 2 \times 100}{m \times f} \quad \text{..... (2)}$$

In which:

X is the content of D_2 (or D_3) in test sample, X, of which the unit is µg/100g ;

C_s is the concentration of vitamin D_2 or D_3 test solution resulted from the standard curve, of which the unit is µg/mL;

m is the mass of sample, of which the unit is gram (g);

f is the recovery coefficient;

Note: the content of Vitamin D in test sample should be the sum of Vitamin D_2 and D_3.

The calculation result of Vitamin D should be expressed as the mean value of two impendent tests and accurate to three decimal places.

7.3 Calculation of Vitamin E: The content of Vitamin E in test sample, should be calculated as formula (3):

$$X = \frac{c_s \times 10 / 2 \times 5 \times 100}{m \times 1000} \quad \text{..... (3)}$$

In which,

X is the content of vitamin, of which the unit is mg/100g;

C_s is the concentration of vitamin E test solution resulted from the standard curve, of which the unit is µg/mL;

m is the mass of sample, of which the unit is gram (g);

The calculation result of Vitamin E should be expressed as the mean value of two independent tests and accurate to three decimal places.

8. Precision:

The absolute difference of results of two individual determinations under repeated condition should not be over 5 % of the arithmetic mean for Vitamins A and E, and 10% for Vitamin D.

9. Others

The detection limit under this standard: vitamin A: 1 µg/100 g; vitamin E: 10.00 µg/100 g; Vitamin D: 0.20 µg/100 g

Annex A

(Normative Annex)

Calibration method for concentration of standard solutions

Vitamins A, D, and E standard stock solutions should be calibrated after preparation, of which the detailed operations are as follows:

Pipet several microliters of Vitamins A, D, and E standard stock solutions respectively into cuvettes containing 3.00 mL of ethyl alcohol; determine the light absorbance according to given wavelengths under the conditions listed in Table 1; the concentration of the vitamin should be calculated as the formula below:

Table 1

Standard substances	The amount of standard stock solution added in (μL)	Specific absorbance $E_{cm}^{1\%}$	Wavelength λ (nm)
Retinol (A)	V	1835	325
α- tocopherol(E)	V	71	294
Ergocalciferol (D_2)	V	485	264
Cholecalciferol (D_3)	V	462	264

The concentration should be calculated according to the following formula (4):

$$C = \frac{A}{E} \times \frac{1}{100} \times \frac{3.00}{V \times 10^{-3}} \quad \text{.....(4)}$$

In which:

C is the concentration of Vitamin A, D, and E, of which the unit is gram per milliliter (g/mL);

A is the average ultraviolet light absorbance of Vitamin A, D, and E;

V is the amount of standard stock solution added in, of which the unit is microliter (μL);

E is the 1% specific light coefficient of Vitamin A, D, and E;

$\frac{3.00}{V \times 10^{-3}}$ is the dilution times of standard stock solution.

Annex B

(Informative annex)

Liquid chromatogram of standard test sample

B.1 The liquid chromatogram of standard Vitamin A, E,D_3, and D_2

The liquid chromatogram of standard Vitamin A, E, D3, and D_2 products see Fig B.1-B.4 respectively.

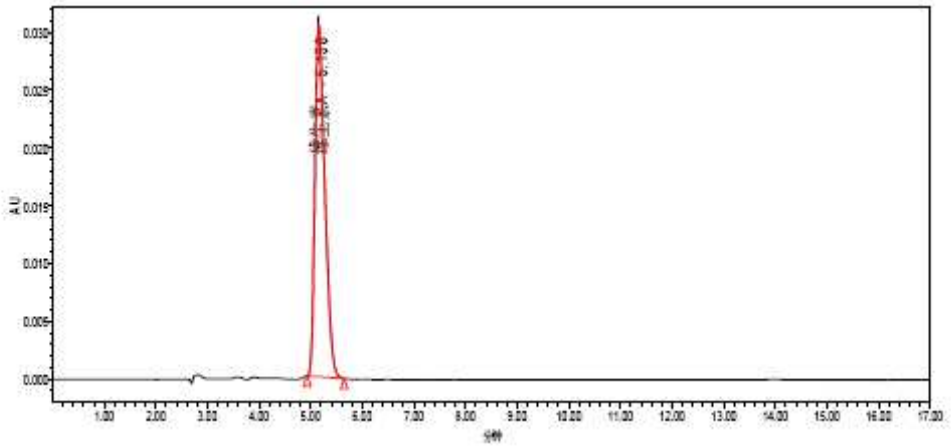

Fig. B.1 liquid chromatogram of standard Vitamin A product

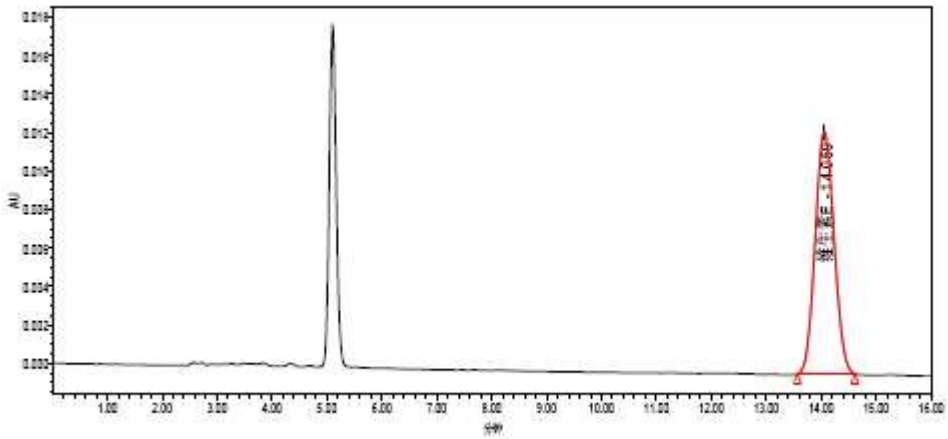

Fig. B.2 liquid chromatogram of standard Vitamin E product

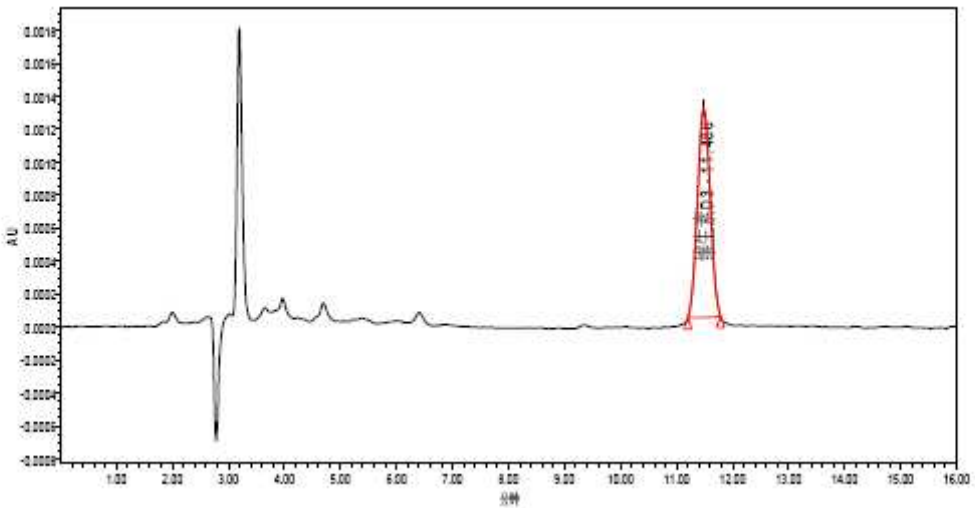

Fig. B.3 liquid chromatogram of standard Vitamin D_3 product

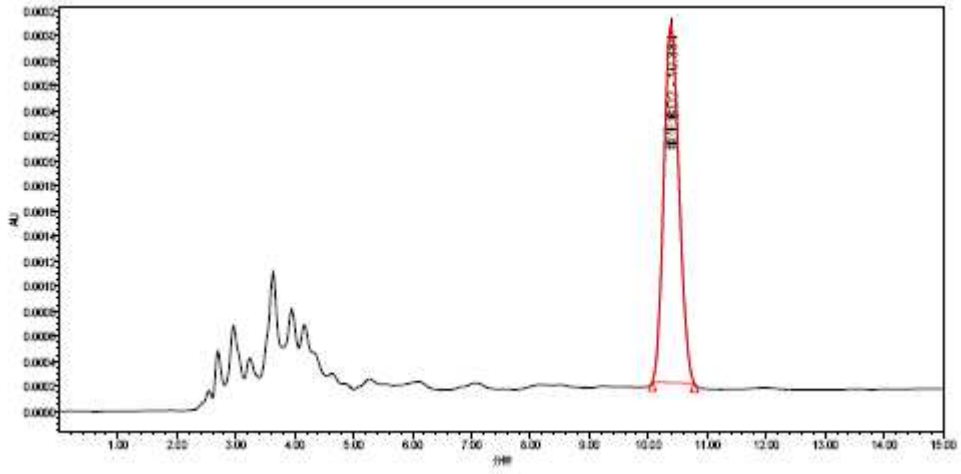

Fig. B.4 liquid chromatogram of standard Vitamin D_2 product