



**NATIONAL STANDARD OF THE
PEOPLE'S REPUBLIC OF CHINA**

GB 1903.44-2020

**National food safety standard - Food Nutritional Fortification
Substance - Hydroxocobalamin**

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1 Scope

This Standard is applicable to the food nutritional fortification substance hydroxocobalamin, which is produced by the fermentation method of *Ensifer adhaerens* through purifying and drying.

2 Molecular Formula, Structural Formula and Relative

Molecular Mass

2.1 Molecular formula

C₆₂H₈₉CoN₁₃O₁₅P

2.3 Relative molecular mass

1346.38 (according to 2018 international relative atomic mass)

Appendix A Test Methods

A.1 General rules Unless otherwise specified, only reagents confirmed to be analytically pure and Class-

1 water specified in GB/T 6682 are used in the analysis. The standard titration solutions, standard solutions for impurity determination, preparations and products that are used in the test methods shall be all prepared in accordance with the provisions of GB/T 601 and GB/T 602, when other requirements are not specified. The used solution refers to an aqueous solution when it is not specified which solvent is used for preparation.

A.2 Identification test A.2.1 Reagents and solutions A.2.1.1 Potassium bromide.

A.2.1.2 Potassium pyrosulfate.

A.2.1.3 Hydrochloric acid.

A.2.1.4 Glacial acetic acid.

A.2.1.5 Sodium acetate.

A.2.1.6 Phenolphthalein indicator solution: 0.1g of phenolphthalein is dissolved in 95% ethanol and make a constant volume to 100mL.

A.2.1.7 Sodium hydroxide solution: 2mol/L. Take 40g of sodium hydroxide; dissolve in water and make constant volume to 500mL.

A.2.1.8 Acetic acid solution: 1mol/L. Pipette 58.8mL of glacial acetic acid in a 1L volumetric flask and make constant volume by water.

A.2.1.9 1-nitroso-2-naphthol-3,6-disulfonate sodium solution: Dissolve 1g of 1-nitroso-2-naphthol-3,6-disulfonate sodium in an appropriate amount of water; transfer to a 100mL volumetric flask and make constant volume with water.

A.2.2 Infrared spectra analysis The infrared spectra analysis is performed according to 5.2.2 of GB/T 6040-2002. The infrared absorption spectrum of the specimen shall be consistent with the standard spectrum of hydroxocobalamin hydrochloride. The standard infrared spectrum of hydroxocobalamin hydrochloride is shown in Figure B.1 of Appendix B.

A.3.2.8 Standard solution: According to the purity conversion, accurately take an appropriate amount of hydroxocobalamin hydrochloride standard product to make it contain hydroxocobalamin hydrochloride 0.02g, accurate to 0.0001g. And use the diluent (A.3.2.7) to dilute it into a 200mL volumetric flask; then make constant volume and use it as a standard solution. Filter it by a 0.45um microporous membrane before use. Parallel specimens are required.

A.3.2.9 Specimen solution: Accurately take 0.02g (calculated according to the sum of the moisture and residual solvent) of the specimen, accurate to 0.0001g; and use the diluent (A.3.2.7) to dissolve and make constant volume to 200mL as the specimen solution; filter with 0.45um microporous membrane before use. Parallel specimens are required.

A.3.3 Apparatus A.3.3.1 Electronic balance with a sensitivity of 0.1 mg.

A.3.3.2 High performance liquid chromatograph.

A.3.4 Reference chromatographic conditions A.3.4.1 Detector: UV detector or diode array detector.

A.3.4.2 Detection wavelength: 351nm.

A.3.4.3 Chromatographic column: Take two columns of alkoxy silica gel (column length 10cm, inner diameter 4.6mm, large pores 2um, mesopores 13nm) with extremely high purity as fillers connected in series or equivalent columns.

A.3.4.4 Column temperature: 30°C.

A.3.4.5 Sampling volume: 20 μ L.

A.3.4.6 Mobile phase: A is methanol, B is buffer solution (A.3.2.6), and C is water. The gradient elution conditions are shown in Table A.1.

- peak area of standard solution;

CU - concentration of hydroxocobalamin in the specimen solution, in mg/mL;

- molar mass of hydroxocobalamin hydrochloride, 1382.8, in g/mol.

A.3.8 Precision The absolute difference between two independent determination results obtained under repeatability conditions shall not exceed 2% of its arithmetic mean.

A.4 Determination of related substances (by anhydrous and solvent-free)

A.4.1 Reagents and solutions A.4.1.1 Methanol: chromatographically pure.

A.4.1.2 Sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$).

A.4.1.3 Phosphoric acid.

A.4.1.4 Buffer solution: Take 15.6g of sodium dihydrogen phosphate; pour it into a 1000mL volumetric flask through a funnel; add a small amount of water to dissolve;

and add water to the mark. Shake well, filter through 0.45 μ m microporous membrane;

adjust the pH to 3.0 with 1:100 phosphoric acid solution; and put it in an ultrasonic water bath to degas for 10min.

A.4.1.5 Specimen dissolving solution: Accurately measure 820mL of water filtered through a 0.45 μ m microporous filter membrane; take 100mL of buffer solution (A.4.1.4)

and 80mL of methanol to mix; shake well; and put it in an ultrasonic water bath to degas for 10min.

A.4.1.6 System suitability solution: 0.75mg/mL. After conversion of purity, accurately take an appropriate amount of hydroxocobalamin hydrochloride standard product (A.3.2.4), and dissolve it by the specimen dissolving solution (A.4.1.5) to a 50mL volumetric flask; make the concentration is 0.75mg/mL as the system suitability solution.

A.4.1.7 Quantitative solution: 7.5 μ g/mL. Respectively accurately pipette 0.5mL of the system suitability solution (A.4.1.6); and use the specimen dissolving solution (A.4.1.5)

to dissolve and make constant volume to a 50mL volumetric flask.

A.4.1.8 Limited solution: 0.75 μ g/mL. Accurately pipette 5mL of a quantitative solution (A.4.1.7); and dissolve with the specimen solution (A.4.1.5) and make constant volume to a 50 mL volumetric flask as the limited solution.

A.4.1.9 Specimen solution: 0.75mg/mL. Accurately take 0.0375g (after calculating A.5.4

Reference chromatographic conditions A.5.4.1 Chromatographic column: Capillary column (30m×0.53mm×3.0μm, the fixative solution is 6% cyanopropyl phenyl-94% polydimethylsiloxane), or equivalent column.

A.5.4.2 Column temperature: 40°C, keep 13min.

A.5.4.3 Detector temperature: 250°C; inlet temperature: 140°C.

A.5.4.4 Carrier gas: N₂; column flow: 4mL/min; air flow: 400mL/min; hydrogen flow: 60mL/min; makeup gas flow: 25mL/min; split ratio: 2:1.

A.5.4.5 Sampling mode: Headspace injection, with an injection volume of 1.0mL.

A.5.4.6 Conditions of headspace sampler: Headspace bottle equilibrium temperature 80°C, equilibrium time 60 min; temperature of quantitative loop 100°C; temperature of transfer line 100°C.

A.5.5 System suitability test Carry out the system suitability test before the specimen test; use the acetone standard use solution (A.5.2.2) for 5 consecutive injections for repeatability test; and calculate the relative standard deviation (RSD) of the peak area. The relative standard deviation (RSD) of repeatability test is required to be no more than 10.0%. At the same time, record the number of theoretical plates of the acetone peak in the first injection of acetone standard use solution (A.5.2.2). The number of theoretical plates shall be no less than 5000. After the conditions are met, the normal test of the specimen is carried out; if it is not met, the system suitability test is continued after the cause is found out, and the specimen test is carried out after passing the system suitability test. Take the acetone standard use solution (A.5.2.2) for injection for each 5 batches of specimens and before the end of the test; and calculate it together with the peak area of the first 5-needle system suitability and the acetone standard peak area confirmed each time in the middle. The relative standard deviation (RSD) of the repeatability test is no more than 10.0%; and the number of theoretical plates of the acetone peak in the acetone standard use solution (A.5.2.2) shall be no less than 5000. If the above conditions are not met, the test data in between is invalid. Re-test the system suitability, and re-test after the system suitability is qualified. The system suitability is valid for 48h.

A.5.6 Analysis steps After the system applicability 5-needle repeatability is qualified, one needle of water sample is injected; and then 2 needles of reference solution (A.5.2.4) and 2 needles of specimen solution (A.5.2.3) are injected in sequence. The prepared reference solution (A.5.2.4) and specimen solution (A.5.2.3) are equilibrated at 80°C for 60min; and 1.0 mL of headspace gas is injected into the sample. Measure under the above chromatographic conditions and record the chromatogram.