A User's Guide

to the new….

International Calibration Standards

for HPLC Analysis of Isomerized & Reduced Isomerized α-Acids

Introduction

International Calibration Standards (ICS) for isomerized and reduced isomerized α-acids are stable and reliable compounds for the calibration of HPLC analysis. Nevertheless, it is recommended that the following procedures be adhered to in regard of their storage and use.

Storage

Store the standards in their original vials, in a freezer set to below – 15°C (+ 5°F), preferably in a desiccator, or otherwise protected from moisture by sealing inside an airtight tin or bottle containing indicating silica gel.

Under these low temperature conditions, it is predicted that the standards will be stable for at least 2 years (DCHA-Iso) or 4 years (DCHA-Rho, DCHA-Hexa, Tetra).

Handling and Use

Warm up

Before opening the desiccator or other outer container, allow time for the contents of the vial to reach room temperature. In most cases, this may conveniently be achieved by allowing the container to stand overnight at room temperature.

Open the outer container and immediately check that the vial is securely closed. Especially in humid conditions, warm the vial in the hand for about 1 minute, inverting it several times to mix the contents, then tapping on the bench to return the standard to the base of the vial.

Weighing

Do not open the vial until you are ready to weigh out the standard. (Refer to Recommended HPLC Method, Item 4.1 for weighing procedure).

Immediately after the standard has been weighed out, close the vial firmly and replace in the desiccator or other outer container. Close the outer container and replace in the freezer.

Preparation of Stock Solution

ICS standards may be made up in one of three solvents:

1. Acidic methanol (0.5 mL 85% H_3PO_4 made up to 1000 mL with HPLC grade methanol)

   Gives best stability – specified by EBC 7.9 and generally recommended, especially for DCHA-Iso.

2. Methanol (HPLC grade)

   Convenient – but should only be used if the standard is to be discarded after same day use. (N.B. DCHA-Iso is not stable in methanol at room temperature. Use acidic methanol or mobile phase).

3. Mobile phase

   Causes no disturbance to the baseline during analysis and often improves peak resolution – recommended if unknown samples are similarly handled. However, it is for the user to determine the stability of standard prepared in this way.

For details of standard solution preparation, see under Recommended HPLC Method, Item 4.

(N.B. Note that greatest accuracy is achieved by avoiding the need to make secondary dilutions).

Storage of Stock Solution

After use, store the stock solution in a freezer, preferably below - 10°C. In acidic methanol, all the standards are stable for at least 1 month if protected from evaporation and light.

For convenience, it is recommended that users consider dispensing the stock solution immediately into HPLC glass vials, stoppering these and storing in the freezer. (In most cases, 100 mL makes about 40 vials).

WARNING: ***It is recommended to use only clear glass vials and volumetric flasks, as certain brown glassware has been found liable to induce instability, at least in solutions of DCHA-Iso standard.*** (This is possibly due to the presence of iron salts in the glass).

Chromatography

The ICS standards are supplied with sample chromatograms and the individual compounds are in most cases identified.

It is believed that these standards are suitable for use as calibration standards in several different elution solvent systems and for the various proportions of isomers (*cis* & *trans)* and major homologs (co, n & ad forms) normally to be found in hop products, worts and beers. However, since the extinction coefficients for individual compounds may differ in relative terms according to the solution conditions, the user is advised that use of different solvent systems may accordingly give different results.

Furthermore, the user is advised that for each standard the assignment of purity and % content of co-homologs was determined in conjunction with the separation afforded by the method EBC 7.8, using only Eluent B as an isocratic mobile phase. Particularly, it should be noted that this method employs a mobile phase based on methanol/water/H_3PO_4 with peak detection at 270 nm. Thus, use of non-acidic eluents and/or detection at other wavelengths is more likely to give erroneous results.

Users should also note that resolution is often substantially improved by the addition of EDTA to the mobile phase. However, experience shows that this is not always the case and some HPLC systems appear to react badly to EDTA, possibly as a consequence of metal ions being stripped from the hardware. Again, it is left to the user to determine whether EDTA is a beneficial additive to the mobile phase.

Recommended HPLC Method

The following method (essentially EBC 7.9) is recommended as a general, isocratic method for determination of all types of isomerized and reduced isomerized α-acids (but not necessarily as mixtures of different types):

1. Equipment

1.1 Balance (accurate to at least 0.1 mg)

1.2 HPLC chromatograph (pump, solvent filter, injection valve, UV detector, integrator)

1.2 Column Heater (optional) set at 35 or 40°C and holding to ±1°C.

1.3 Injection Loop (preferably 10 µL, not more than 20 µL)

1.4 Analysis Column (ODS, 250 x 4.6 mm, C18, 5µ, Nucleosil or equivalent; or, 125 x 4.6 mm Nucleodur 100-5 C18 ec or equivalent*)

1.5 Precolumn (optional)

1.6 Sonic bath (optional)

** Some analysts prefer to use 100 x 4.6 mm columns with 3 or 4µ packing, which improves the resolution of the shorter column.*

2. Reagents

2.1 Water (HPLC Grade)

2.2 Methanol (HPLC Grade) ("MeOH")

2.3 Orthophosphoric acid (H_3PO_4), 85% (Analytical Grade)

2.4 0.1M EDTA, Na_2 or Na_4 salt (Analytical Grade). *(Na_4 salt is easier to dissolve)*

2.5 Acidic MeOH (optional): add 0.5 mL H_3PO_4 (85%) to 1000 mL of MeOH

3. Mobile Phase

3.1 Prepare the following solution:

750 mL MeOH
240 mL Water
10 mL H_3PO_4 (85%)
1 mL 0.1M Na_2 (or Na_4) EDTA (Optional*)

** In some HPLC systems the addition of EDTA sharpens the peaks by preventing trace metal catalyzed oxidation of iso-α-acids.*

4. Preparation of Stock Solution

With reference to the detailed Handling and Use instructions already given above:

4.1 Being accurate to at least 0.1 mg, weigh out the standard into a glass weighing boat (18 – 20 mg for DCHA standards, 12 – 14 mg for Tetra standard).

4.2 Transfer the standard into a 100 mL*, clear glass, Grade A volumetric flask, then using the chosen solvent (MeOH, Mobile Phase or Acidic MeOH), rinse any remaining powder down into the flask. Add further solvent to a total of about 40 mL and swirl or sonicate until the standard is fully dissolved. Finally, make up to the mark at 20°C, stopper and mix by inversion at least four times. Protect the solution from light (e.g. by wrapping the flask in aluminium foil).

** If using a 20µL injector loop, then transfer into a 200 mL flask.*

4.3 If desired, dispense the stock solution immediately into HPLC glass vials and store below - 10°C.

(N.B. Because methanol has a high coefficient of thermal expansion, equilibrate and maintain the solution to a constant temperature (e.g. ambient) before injecting into the chromatograph at this same temperature. (Ensure that all unknown sample solutions are similarly handled, too)).

5. Chromatography *(See also EBC 7.9 and the specific notes supplied with the standard used)*

5.1 Set the detector to 270 nm.

(N.B. At this wavelength the extinction coefficients of cis- and trans-iso-α-acids are virtually identical in the mobile phase of this method).

5.2 Start the pump. (Recommended flowrate: 1.0 – 1.4 mL/min, depending on temperature and equipment).

5.1 If not already done, clean the column by running MeOH until contaminants are removed and the baseline is steady. Then, condition the column to the mobile phase by running this under the analytical conditions until the baseline is steady at low attenuation.

5.2 Set a sufficiently low figure for the area reject on the integrator such that all the significant minor peaks are integrated but noise is rejected.

5.2 Inject the standard solution.

5.3 Run the chromatogram until all minor peaks are eluted.

5.4 Check that all obvious peaks are detected as such and sensibly integrated.

(If possible, set the integrator to display the baseline for each peak. If the baseline does not appear to be appropriate, then consider resetting the integration parameters as per instructions given in the instrument manual).

5.4 Repeat the injection at least once to establish reproducibility.

6. Calibration

6.1 With reference to the specific guidance notes for the particular standard, identify the peaks by comparison against the standard chromatogram supplied.

6.2 Selecting only the peaks corresponding to those (major) compounds against which the % composition of the standard has been declared, determine the total area counts/mg of isomerized or reduced isomerized α-acids injected.

6.3 Calibrate the integrator according to manufacturer's instructions, calculating a single response factor to be applied to all the above-selected, isomerized or reduced isomerized α-acids peaks.

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