Supplementary Information for

Development of a Primary Reference Material of Natural C-Reactive Protein: Verification of its natural pentamer structure and certificated by two isotope dilution mass spectrometry

Gel electrophoresis

Take CRP solution (1.2 mg/mL) from -20 ℃ freezer, then equilibrate at room temperature. Add 2 μL CRP solution into 0.2 mL EP tube. Mix the sample and the loading buffer in the same proportion, and boil in a water bath at 100°C for 10 minutes;

Prepare loading buffer: 1.25mL 0.5mol/L Tris-HCl, pH 6.8, 3.0mL glycerol, 0.2mL 0.5% bromophenol blue, 5.5 mL Milli-Q; store at -20°C, dilute 1 time when used.

Prepare Coomassie brilliant blue staining solution: Coomassie brilliant blue R-250 2.5g, methanol 400mL, glacial acetic acid 100mL, Milli-Q 500mL.

Prepare Coomassie brilliant blue decolorizing solution: methanol 400mL, glacial acetic acid 100mL, Milli-Q 500mL.

Insert the gel into the electrophoresis tank, pour the electrophoresis buffer until the buffer overflows the inner and outer tank; add the sample and Marker separately Sample tank; set the electrophoresis condition to 110V; when the indicator moves to about 1cm from the bottom of the gel plate, stop electrophoresis. Take out the gel plate, carefully peel off the gel, stain it in 0.25% staining solution for about 30min, change to decolorizing solution, shake slowly, and replace the decolorizing solution after 15min, 30min, 1h, and 12h until the gel background is

clear and clean.

Operating conditions of tandem triple-quadrupole MS

Table S1. Operating conditions of tandem triple-quadrupole MS

Agilent 6410 Triple-quadrupole MS parameters	
Ion source	ESI
Capillary voltage	4 kV
Dry gas	8 L/min
Gas temperature	350 ℃

Homogeneity results and uncertainty evaluation for the CRP sample

Table S2. Homogeneity results for the CRP sample

Source of difference	SS	df	MS	*F*	*P*-value	*F* crit
Between groups	1.228206	10	0.122821	1.489665	0.208694	2.296696
Within groups	1.813867	22	0.082448			
Total	3.042073	32				

When evaluating u_h, two factors need to be considered: The first is the uniformity of the standard material, which is measured by the estimated standard deviation s_{bb} between bottles, which reflects the degree of unevenness between sampling bottles; the second is the detection method. The repeatability level is the contribution of the repeatability standard deviation s_r of the test method to the standard deviation between bottles, measured by the estimator uh. If u_h is less than s_{bb}, it indicates that the repeatability of the detection method is very good, and it can sensitively respond to small differences between bottles, that is, unevenness. In this case, the standard deviation estimate s_{bb} between bottles is used to evaluate the contribution of sample unevenness to the total uncertainty. If u_h is greater than s_{bb}, the repeatability level of the detection method is not good enough, causing the in-bottle variance to be greater than the inter-bottle variance; in this case, the estimator u'_{bb} is used to evaluate the contribution of sample unevenness to the total uncertainty. Combining the above two situations, in order to make a safe estimate of the uncertainty caused by the inhomogeneity of the reference material, the uniformity test data can be used to calculate the two statistics of u_h and s_{bb}, and the larger of the two statistics can be used as the opposite. Estimation of uncertainty caused by inhomogeneity. The specific calculation method and formula are as follows:

$$s_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}} \qquad \text{(s1)}$$

$$s_r = \sqrt{MS_{within}} \qquad \text{(s2)}$$

$$u_h = \sqrt{\frac{MS_{within}}{n}\sqrt{2/vMS_{within}}} \qquad \text{(s3)}$$

The uncertainty caused by the inhomogeneity of CRP sample is 1.116 mg/L.

Stability results and uncertainty evaluation for the CRP sample

This study used amino acid hydrolysis method to test the stability of CRP content. Perform t-distribution test on the measurement results, that is, if the t_i value of the i test is less than the critical value $t_{0.05,\ (i-2)}$, it indicates that the characteristic value of the test standard substance has no significant change.

That is, the t_i value is calculated as follows: $t_i = \frac{|b|}{s(b)}$ (s4)

Linear regression slope： $b = \frac{\sum_{i=1}^{n}(X_i - \overline{X})(Y_i - \overline{Y})}{\sum_{i=1}^{n}(X_i - \overline{X})^2}$ (s5)

Linear regression intercept： $b_0 = \overline{Y} - b_1\overline{X}$ (s6)

Linear regression standard deviation of each point： $s = \sqrt{\frac{\sum_{i=1}^{n}(Y_i - b_0 - b_1X_i)^2}{n-2}}$ (s7)

Uncertainty of slope b： $s(b) = \frac{s}{\sqrt{\sum_{i=1}^{n}\left(x_i - \overline{x}^2\right)}}$ (s8)

In the long-term stability test of CRP, b_1 is -0.004; b_0 is 54.788; ss is 0.145; sb is 0.019; v is 5.000; t_{limit} is 2.571; The slope is not significant, and no instability trend is observed. The uncertainty contribution caused by long-term stability is: u=0.427 mg/L.

Molecular weights of the peptides as determined by mass spectrometry

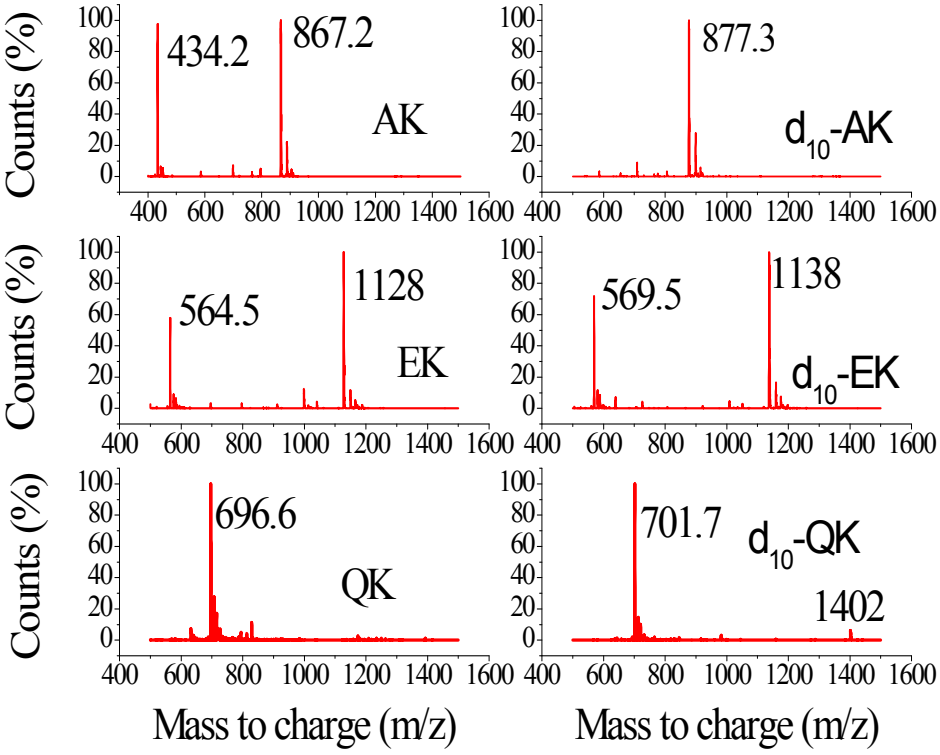

Fig. S1 Molecular weights of the peptides as determined by mass spectrometry

Uncertainty of CRP hydrolysis

The uncertainty of method reproducibility calculated form the result of valine, leucine, isoleucine, and phenylalanine were 0.29%, 0.30%, 0.40%, and 0.36%, respectively.

Uncertainty arose from each standard amino acid CRMs were calculated form the uncertainty provide in certificate of CRMs (Table S3). The coverage factor *k* was determined to be 2, corresponding to a 95% confidence level, half of the relative expanded uncertainty were taken into account.

Table S3. Values and uncertainties of amino acid CRMs

	Name	Value (%)	Expanded uncertainty (%), k=2
GBW(E)100055	Purity of valine	99.4	1.6
GBW(E)100057	Purity of Isoleucine	99.4	1.5
GBW(E)100058	Purity of Leucine	99.5	1.5
GBW(E)100061	Purity of Phenylalanine	99.9	1.5
GBW(E)100084	Purity of Proline	99.0	1.5

Uncertainties in the purities of the valine (0.8%), leucine (0.75%), isoleucine (0.75%), proline (0.75%) and phenylalanine (0.75%)

Based on the previous work and previous experiment result in the international comparison, the relative uncertainty introduced by the hydrolysis efficiency is estimated to be 1%.