Quilpue, Chile, February 6, 2013

To whom it may concern,

Re: Clarification Letter of Saponins Detection Test in Low Concentration Samples

The analytical method for saponin determination, I-PR-7.6-27 (C-18 UPLC method for total saponin determination in Quillaja extracts), was developed for determination of saponin concentration of solutions with a minimum of 2.13 grams per liter of saponins (2130 ppm). In order to determine low concentrations of saponins in finished beverages (less than 50 ppm), detection limits must be enhanced at least 40 fold. The sequence to achieve this is the following:

1) Concentrate the sample (using a rotary evaporator)
2) Enhance the detection limits of the analytical device
3) The solvents used must be changed in order to enhance the performance of the chromatographic system.

In order to enhance the detection limit of the analytical device:

1) Change the chromatograph detection cell to an ultra-sensitive detection cell. This allows the instrument response signal to be enhanced 3 fold.
2) Increase the injection volume from 1.5 µL to 5.0 µL. This increases the injection mass capacity 3.33 times.
3) To minimize the slope effect of the base line due to solvent the gradient, change the organic acid to a mineral acid, this reduces the noise level achieving a more stable base line.

With these adjustments over the original method, the detection capacity can be enhanced in such a way that, in the case of the standard curves, the chromatographic response is able to measure saponin solutions as low as 25 ppm.

Please do not hesitate to contact me if you have any other questions.

[redacted]

Director
Natural Response

Protocol

Saponins Detection Test in Low Concentration Samples

1. Prepare mobile phase A and B replacing the formic acid with ortho phosphoric acid.

2. Change UPLC II detection cell for a high sensitivity one (Sensitivity High Flow Cell 2400 nL, 25-mm path length serial: 205015005)

3. Prepare a Super Sap solution concentration of 0.24 g / L, from 40 g / L standard calibration solution as follows:

   Using a 20-100 uL range micropipette, take 60 uL of the 40 g / L saponin standard solution and transfer to a 10 ml volumetric flask. Dilute with HPLC grade water up to the mark. The final solution will be at 0.24 g / L of saponin (labeled as Pattern 240 ppm).

4. Prepare a calibration curve from the above solution of 240 ppm using the following table:

Solution of Super Sap 240 ppm (uL)	HPLC water (uL)	Total Volumen (uL)	Concentration (g/L)	Concentration (ppm)
25	975	1000	0,006	6
50	950	1000	0,012	12
100	900	1000	0,024	24
200	800	1000	0,048	48
300	700	1000	0,072	72
400	600	1000	0,096	96
500	500	1000	0,12	120
600	400	1000	0,144	144
700	300	1000	0,168	168
800	200	1000	0,192	192
900	100	1000	0,216	216
1000	0	1000	0,24	240

5. Inject 5.0 uL of each of the solution from the above table to verify linearity (R>0.98)

6. After comfirming the step above, inject your low concentration product sample.