

Appendix 8 Relative Sweetness Intensity Method

Sweetness report comparison with Sucrose

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1 Test purpose

Determine the sweetness of enzyme modification and refined enzyme modification

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Qufu Shengren Pharmaceutical Co., Ltd., Sunwin Stevia International, & NuNaturals, Inc.

4/25/19

2 Testers: Yang Haiwei, Jia Yuqing, Kong Linghua, Kong Min, Yuan Hui, Yuan Huijun, Zheng Honglei, Yan Aiping, Wu Min, Peng Yu

3 Test methods

The Sweetness tests of Enzyme Treated stevia and Refined Enzyme treated stevia are based on the GB 8270 food additive stevioside: Weigh 2 g of sucrose, add 100 ml of water, and make a 2% sucrose solution. Another enzyme treated stevia or refined Enzyme treated stevia (2/n) g, add water 100ml dissolved. Compare the taste of the two solutions. When the enzyme treated stevia or refined enzyme treated stevia is equivalent to the sweetness of the 2% sucrose solution, the value of n, that is, the enzyme treated stevia or purified enzyme treated stevia is the multiple of sucrose sweetness. The evaluation team consisted of 10 members. These evaluators have already passed the test of taste sensitivity and have also conducted evaluation training on sweetness intensity values. Prior to the assessment, each assessor had conducted an aqueous sugar solution and training using a scoring system. There is a five-minute break between the supplies of each sample, and they are provided with water and salt-free cookies to clean the taste. Then we use the Friedman test table for statistical analysis.

4 Test process

4.1 Weigh 2g of sucrose (No. A) and add 100ml of water to make a 2% sucrose solution. Another batch of enzyme treated stevia 20150401, 0.0182 g, 0.0167g, and 0.0153g (No. B, C, D) was taken and dissolved in 100 ml of water. Ten reviewers evaluated the above solutions and ranked the degree of sweetness. The results are shown in Table 1.

Table 1 The rank of sweetness tested by 10 testers

Batch: 20150401

Tester	Sample				Rank Sum
	A	B	C	D	
1	2.5	1	2.5	4	10
2	2.5	1	2.5	4	10
3	2.5	1	2.5	4	10
4	2.5	1	2.5	4	10
5	2.5	1	2.5	4	10
6	2.5	1	2.5	4	10
7	2.5	1	2.5	4	10
8	2.5	1	2.5	4	10
9	2.5	1	2.5	4	10

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10	2.5	1	2.5	4	10
Rank Sum	25	10	25	40	100

Note: 1, Sweetest, 2, average, And so on. If the same degree occurs, it can be expressed by the average number of the summation.

4.1.1 Friedman inspection

Computational Statistics F

$j=10, p=4, R_1 = 25, R_2 = 10, R_3 = 25, R_4 = 40$

Per

$$x^F = \frac{12}{jp(p+1)}(R_1^2 + R_p^2) - 3j(p+1)$$

$$F = \frac{12}{10 \times 4 \times (4+1)}(25^2 + 10^2 + 25^2 + 40^2) - 3 \times 10 \times (4+1) = 27$$

Since F is greater than the critical value of 7.67 in the table corresponding to $j=10, p=4, a=0.05$, it can be considered that there is a significant difference between the four samples when the significance level is less than or equal to 5%.

4.1.2 Multiple Comparisons and Grouping

If the absolute value of the difference between the rank sums of the two samples is greater than the least significant difference LSD, it can be considered that there is a significant difference between the two.

The least significant difference LSD:

$$LSD = 1.96 \times \sqrt{\frac{10 \times 4 \times (4+1)}{6}} = 11.32$$

At the significant level of 0.05, the differences between A and B, A and D, C and B, and C and D are significant, and the absolute values of the differences between their rank sums are:

$$A-B: |25 - 10| = 15, \quad C-B: |10 - 25| = 15,$$

$$A-D: |25 - 40| = 15, \quad C-D: |25 - 40| = 15,$$

At a significant level of 0.05, there is no significant difference between A and C, and the absolute value of their difference in rank sum is:

$$A-C: |25 - 25| = 0,$$

Therefore, four samples are divided into 3 groups, one group is B, the other group includes A and C, and the third group is D. Therefore, the sweetness of enzyme treated stevia lot 20150401 is 120.

4.2 Weigh 2g of sucrose (No. A) and add 100ml of water to make a 2% sucrose solution. Another batch of enzyme treated stevia batch# 20150402 of 0.0183 g, 0.0168g, 0.0154g (No. B, C, D), were dissolved by adding 100ml water. Ten

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reviewers evaluated the above solutions and ranked them according to the degree of sweetness. The results are shown in Table 2.

Table 2, The rank of sweetness tested by 10 testers

Batch: 20150402

Tester	Sample				Rank Sum
	A	B	C	D	
1	2.5	1	2.5	4	10
2	2.5	1	2.5	4	10
3	2.5	1	2.5	4	10
4	2.5	1	2.5	4	10
5	2.5	1	2.5	4	10
6	2.5	1	2.5	4	10
7	2.5	1	2.5	4	10
8	2.5	1	2.5	4	10
9	2.5	1	2.5	4	10
10	2.5	1	2.5	4	10
Rank Sum	25	10	25	40	100

Note: 1, Sweetest, 2, average, And so on. If the same degree occurs, it can be expressed by the average number of the summation.

4.2.1 Friedman Test

Computational Statistics F

$j=10, p=4, R_1 = 25, R_2 = 10, R_3 = 25, R_4=40$

Based on

$$x_F = \frac{12}{jp(p+1)} (R_1^2 + R_p^2) - 3j(p+1)$$

$$F = \frac{12}{10 \times 4 \times (4+1)} (25^2 + 10^2 + 25^2 + 40^2) - 3 \times 10 \times (4+1) = 27$$

Since F is greater than the critical value of 7.67 in the table corresponding to $j=10, p=4, a=0.05$, it can be considered that there is a significant difference between the four samples when the significance level is less than or equal to 5%.

4.2.2 Multiple comparisons and grouping

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If the absolute value of the difference between the rank sums of the two samples is greater than the least significant difference LSD, it can be considered that there is a significant difference between the two.

Least Significant Difference LSD:

$$LSD=1.96 \times \sqrt{\frac{10 \times 4 \times (4+1)}{6}}=11.32$$

At the significant level of 0.05, the differences between A and B, A and D, C and B, and C and D are significant, and the absolute values of the differences between their rank sums are:

A-B: |25 – 10|=15, C-B: |10 – 25|=15,

A-D: |25 – 40|=15, C-D: |25 – 40|=15,

At a significant level of 0.05, there is no significant difference between A and C, and the absolute value of the difference between their rank sums is:

A-C: |25 – 25|=0,

Therefore, four samples are divided into 3 groups, one group is B, the other group includes A and C, and the third group is D. Therefore, the sweetness of the enzyme modification 20150402 batch is 120.

4.3 Weigh 2g of sucrose (No. A) and add 100ml of water to make a 2% sucrose solution. Another batch of enzyme treated stevia batch#20150403 of 0.0184 g, 0.0167 g, and 0.0153 g (numbers B, C, and D) was added and dissolved in 100 ml of water. Ten reviewers evaluated the above solutions and ranked the degree of sweetness. The results are shown in Table 3.

Table 3 The rank of sweetness tested by 10 testers

Batch:20150403

Tester	Sample				Rank Sum
	A	B	C	D	
1	2.5	1	2.5	4	10
2	2.5	1	2.5	4	10
3	2.5	1	2.5	4	10
4	2.5	1	2.5	4	10
5	2.5	1	2.5	4	10
6	2.5	1	2.5	4	10
7	2.5	1	2.5	4	10
8	2.5	1	2.5	4	10

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9	2.5	1	2.5	4	10
10	2.5	1	2.5	4	10
Rank Sum	25	10	25	40	100

Note: 1, Sweetest, 2, average, And so on. If the same degree occurs, it can be expressed by the average number of the summation.

4.3.1 Friedman test

Computational Statistics F

$$j=10, p=4, R_1 = 25, R_2 = 10, R_3 = 25, R_4=40$$

Based on

$$x_F = \frac{12}{jp(p+1)} (R_1^2 + R_2^2 + R_3^2 + R_4^2) - 3j(p+1)$$

$$F = \frac{12}{10 \times 4 \times (4+1)} (25^2 + 10^2 + 25^2 + 40^2) - 3 \times 10 \times (4+1) = 27$$

Since F is greater than the critical value of 7.67 in the table corresponding to j=10, p=4, a=0.05, it can be considered that there is a significant difference between the four samples when the significance level is less than or equal to 5%.

4.3.2 Multiple comparisons and grouping

If the absolute value of the difference between the rank sums of the two samples is greater than the least significant difference LSD, it can be considered that there is a significant difference between the two.

Least Significant Difference LSD:

$$LSD = 1.96 \times \sqrt{\frac{10 \times 4 \times (4+1)}{6}} = 11.32$$

At the significant level of 0.05, the differences between A and B, A and D, C and B, and C and D are significant, and the absolute values of the differences between their rank sums are:

$$A-B: |25 - 10| = 15, \quad C-B: |10 - 25| = 15,$$

$$A-D: |25 - 40| = 15, \quad C-D: |25 - 40| = 15,$$

At a significant level of 0.05, there is no significant difference between A and C. The absolute value of the difference between the two rank sums is:

$$A-C: |25 - 25| = 0,$$

Therefore, four samples are divided into 3 groups, one group is B, the other group includes A and C, and the third group is D. So the sweetness of the enzyme treated stevia upgrade 20150403 batch is 120.

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4.4 Weigh 2g of sucrose (No. A), add 100ml of water, and make a 2% sucrose solution. Another batch of purified enzyme modification 20150301 batches of 0.0077 g, 0.0074g, and 0.0071g (Nos. B, C, and D) were dissolved in 100 ml of water. Ten reviewers evaluated the above solutions and ranked the degree of sweetness. The results are shown in Table 4.

Table 4 The rank of sweetness tested by 10 testers

Batch: 20150301

Tester	Sample				Rank Sum
	A	B	C	D	
1	2.5	1	2.5	4	10
2	2.5	1	2.5	4	10
3	2.5	1	2.5	4	10
4	2.5	1	2.5	4	10
5	2.5	1	2.5	4	10
6	2.5	1	2.5	4	10
7	2.5	1	2.5	4	10
8	2.5	1	2.5	4	10
9	2.5	1	2.5	4	10
10	2.5	1	2.5	4	10
Rank Sum	25	10	25	40	100

Note: 1 is the sweetest, 2 is the average, and so on. If the same degree occurs, it can be expressed by the average number of the summation.

4.4.1 Friedman Test

Computational Statistics F

$$j=10, p=4, R_1 = 25, R_2 = 10, R_3 = 25, R_4=40$$

Based on

$$x_F = \frac{12}{jp(p+1)} (R_1^2 + R_2^2 + R_3^2 + R_4^2) - 3j(p+1)$$

$$F = \frac{12}{10 \times 4 \times (4+1)} (25^2 + 10^2 + 25^2 + 40^2) - 3 \times 10 \times (4+1) = 27$$

Since F is greater than the critical value of 7.67 in the table corresponding to j=10, p=4, a=0.05, it can be considered that there is a significant

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difference between the four samples when the significance level is less than or equal to 5%.

4.4.2 Multiple comparisons and grouping

If the absolute value of the difference between the rank sums of the two samples is greater than the least significant difference LSD, it can be considered that there is a significant difference between the two.

The least significant difference LSD:

$$LSD=1.96 \times \sqrt{\frac{10 \times 4 \times (4+1)}{6}}=11.32$$

At the significant level of 0.05, the differences between A and B, A and D, C and B, and C and D are significant, and the absolute values of the differences between their rank sums are:

$$A-B:|25 - 10|=15, \quad C-B:|10 - 25|=15,$$

$$A-D:|25 - 40|=15, \quad C-D:|25 - 40|=15,$$

At a significant level of 0.05, there is no significant difference between A and C, and the absolute value of the difference between their rank sums is:

$$A-C:|25 - 25|=0,$$

Therefore, four samples are divided into 3 groups, one group is B, the other group includes A and C, and the third group is D. Therefore, the sweetness of the refined enzyme treated stevia 20150301 lot is 270.

4.5 Weigh 2g of sucrose (No. A) and add 100ml of water to make a 2% sucrose solution. Another batch of purified enzyme modification 20150302 batches of 0.0078g, 0.0075g, and 0.0072g (Nos. B, C, and D) were dissolved in 100 ml of water. Ten reviewers evaluated the above solutions and ranked the degree of sweetness. The results are shown in Table 5.

Table 5 The rank of sweetness tested by 10 testers

Batch: 20150302

Tester	Sample				Rank Sum
	A	B	C	D	
1	2.5	1	2.5	4	10
2	2.5	1	2.5	4	10
3	2.5	1	2.5	4	10
4	2.5	1	2.5	4	10
5	2.5	1	2.5	4	10

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6	2.5	1	2.5	4	10
7	2.5	1	2.5	4	10
8	2.5	1	2.5	4	10
9	2.5	1	2.5	4	10
10	2.5	1	2.5	4	10
Rank Sum	25	10	25	40	100

Note: 1, Sweetest, 2, average, And so on. If the same degree occurs, it can be expressed by the average number of the summation.

4.5.1 Friedman test

Computational Statistics F

$j=10, p=4, R_1 = 25, R_2 = 10, R_3 = 25, R_4 = 40$

Based on

$$x^F = \frac{12}{jp(p+1)}(R_1^2 + R_p^2) - 3j(p+1)$$

$$F = \frac{12}{10 \times 4 \times (4+1)}(25^2 + 10^2 + 25^2 + 40^2) - 3 \times 10 \times (4+1) = 27$$

Since F is greater than the critical value of 7.67 in the table corresponding to $j=10, p=4, \alpha=0.05$, it can be considered that there is a significant difference between the four samples when the significance level is less than or equal to 5%.

4.5.2 Multiple comparisons and grouping

If the absolute value of the difference between the rank sums of the two samples is greater than the least significant difference LSD, it can be considered that there is a significant difference between the two.

The least significant difference LSD:

$$LSD = 1.96 \times \sqrt{\frac{10 \times 4 \times (4+1)}{6}} = 11.32$$

At the significant level of 0.05, the differences between A and B, A and D, C and B, and C and D are significant, and the absolute values of the differences between their rank sums are:

$$A-B: |25 - 10| = 15, \quad C-B: |10 - 25| = 15,$$

$$A-D: |25 - 40| = 15, \quad C-D: |25 - 40| = 15,$$

At a significant level of 0.05, there is no significant difference between A and C, and the absolute value of the difference between their rank sums is:

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A-C: |25 – 25|=0,

Therefore, four samples are divided into 3 groups, one group is B, the other group includes A and C, and the third group is D. Therefore, the sweetness of the refined enzyme treated stevia batch# 20150302 is 270.

4.6 Weigh 2g of sucrose (No. A) and add 100ml of water to make a 2% sucrose solution. Another batch of Refined enzyme treated stevia Batch 20150303 of 0.0076 g, 0.0073 g, and 0.0071 g (Nos. B, C, and D) was added and dissolved in 100 ml of water. Ten reviewers evaluated the above solutions and ranked them according to the degree of sweetness. The results are shown in Table 6. .

Table 6 The rank of sweetness tested by 10 testers

Batch# 20150303

Tester	Sample				Rank Sum
	A	B	C	D	
1	2.5	1	2.5	4	10
2	2.5	1	2.5	4	10
3	2.5	1	2.5	4	10
4	2.5	1	2.5	4	10
5	2.5	1	2.5	4	10
6	2.5	1	2.5	4	10
7	2.5	1	2.5	4	10
8	2.5	1	2.5	4	10
9	2.5	1	2.5	4	10
10	2.5	1	2.5	4	10
Rank Sum	25	10	25	40	100

Note: 1 is the sweetest, 2 is the average, and so on. If the same degree occurs, it can be expressed by the average number of the summation.

4.6.1 Friedman test

Computational Statistics F

$j=10, p=4, R_1 = 25, R_2 = 10, R_3 = 25, R_4=40$

Based on

$$x_F = \frac{12}{jp(p+1)} (R_1^2 + R_p^2) - 3j(p+1)$$

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$$F = \frac{12}{10 \times 4 \times (4 + 1)} (25^2 + 10^2 + 25^2 + 40^2) - 3 \times 10 \times (4 + 1) = 27$$

Since F is greater than the critical value of 7.67 in the table corresponding to j=10, p=4, a=0.05, it can be considered that there is a significant difference between the four samples when the significance level is less than or equal to 5%.

4.6.2 Multiple comparisons and grouping

If the absolute value of the difference between the rank sums of the two samples is greater than the least significant difference LSD, it can be considered that there is a significant difference between the two.

The least significant difference LSD:

$$LSD = 1.96 \times \sqrt{\frac{10 \times 4 \times (4 + 1)}{6}} = 11.32$$

At the significant level of 0.05, the differences between A and B, A and D, C and B, and C and D are significant, and the absolute values of the differences between their rank sums are:

$$A-B: |25 - 10| = 15, \quad C-B: |10 - 25| = 15,$$

$$A-D: |25 - 40| = 15, \quad C-D: |25 - 40| = 15,$$

At a significant level of 0.05, there is no significant difference between A and C. The absolute value of the difference between the two rank sums is:

$$A-C: |25 - 25| = 0,$$

Therefore, four samples are divided into 3 groups, one group is B, the other group includes A and C, and the third group is D. Therefore, the sweetness of the refined enzyme treated stevia Batch# 20150303 batch was 270.

5 Conclusion

The relative sweetness of enzyme treated stevia and sucrose is shown in the table 7

Table 7, Test result of sweetness of enzyme treated stevia

Product	Batch	Parameters	Standard	Result
Enzyme Treated Stevia	20150401	Sweetness	100-150	120
Enzyme Treated Stevia	20150402	Sweetness	100-150	120
Enzyme Treated Stevia	20150403	Sweetness	100-150	120

The relative sweetness of Refined enzyme treated stevia and sucrose is shown in the table 8,

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Product	Batch	Parameter	Standard	Result
Refined Enzyme Treated Stevia	20150301	Sweetness	>260	270
Refined Enzyme Treated Stevia	20150302	Sweetness	>260	270
Refined Enzyme Treated Stevia	20150303	Sweetness	>260	270

6 Appendix

- 6.1 GB 8270-1999 Food Additive, steviol glycosides
- 6.2 GB 8270-2014 Food Additive, Steviol Glycosides
- 6.3 GB/T 12315-2008 Sensory Analysis, Methodology, Sorting Method

Appendix 9 TasteRight Enzyme Treated Stevia Stability Report

Test Report on Influence Factors of Enzyme Treated Stevia (Batch: 20150401)

Test Conditions	Days	Test Items			
		Appearance	Moisture-absorption Weight gain (%)	Loss on Drying (%)	Assay (%)
	0	White Powder, Odorless or Characteristic	—	3.02	96.16
High temperature (60°C)	5	White Powder, Odorless or Characteristic	—	3.04	96.45
	10	White Powder, Odorless or Characteristic	—	2.98	96.10
Light (4500lx)	5	White Powder, Odorless or Characteristic	—	2.99	96.22
	10	White Powder, Odorless or Characteristic	—	3.03	96.08
High Humidity (RH92.5%)	5	White Powder, Odorless or Characteristic	8.2%	-	95.81
High Humidity (RH75%)	5	White Powder, Odorless or Characteristic	7.9%	—	96.09

1. This test is conducted under more intense conditions than the accelerated test. Its purpose is to explore the inherent stability of the product and to understand the factors affecting its stability as the production process, packaging.

Storage conditions provide scientific basis.

2.1 The batch of samples is placed in a thermostatic drying oven, placed at a temperature of 60°C for 10 days, sampled on the 5th and 10th days, and examined according to the requirements of the stability inspection item.

The results are compared with 0 months and the results are shown in the above table. The test data show that there is no obvious change in the test results, and the test at 40°C is no longer performed.

2.2 The batch of test samples shall be placed in a sealed container of constant humidity and placed at 25 °C under a relative humidity of 90% ± 5% for 10 days. Sampled to be taken on the 5th and 10th days.

The requirements of the inspection project were compared with the 0-month results. The results are shown in the above table. The test data showed that there was no significant change in the content test results. At a relative humidity of 90% ± 5% at 25°C

Placed for 10 days under the conditions, and sampled on the 5th and 10th days, according to the requirements of the stability inspection project requirements, compared with 0 months results, the results are shown in the above table, the test data show that there was no significant change in the content test results.

Therefore, the moisture of the product can be absorbed to saturated status in 24 hours, so no need to observe the content change results.

Even though, we also have done the 10 days observation for your reference only, but not supposed to shown on above table.

3 The batch of test specimens were placed in a light box with a fluorescent lamp under an illumination of 4500 lx, placed for 10 days, and sampled on the 5th and 10th days.

The test is required to be compared with the 0-month results. The results are shown in the above table. The test data show that there is no significant change in the test results.

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Accelerated test report of Enzyme treated Stevia

Batch	Test (Mo's)	Appearance	Loss on Drying (%)	Ashes (%)	Assay (%)	Microbial Limit
20150401	0	White Powder	3.02	0.09	SG: 96.16%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	1	White Powder	3.01	0.09	SG:96.44%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	2	White Powder	3.02	0.09	SG:96.11%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	3	White Powder	3.06	0.09	SG:96.05%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	6	White Powder	3.01	0.09	SG:96.19%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
20150402	0	White Powder	3.16	0.09	SG:96.47%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	1	White Powder	3.15	0.09	SG:96.56%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	2	White Powder	3.18	0.09	SG:97.75%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	3	White Powder	3.14	0.09	SG:96.20%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	6	White Powder	3.15	0.09	SG:96.68%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
20150403	0	White Powder	3.25	0.09	SG:96.19%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	1	White Powder	3.25	0.09	SG:96.09%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	2	White Powder	3.24	0.09	SG:96.43%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	3	White Powder	3.28	0.09	SG:95.94%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	6	White Powder	3.19	0.09	SG:96.90%	TABC<10 cfu/g Yeast/Mold<10 cfu/g

1. This test is conducted under accelerated conditions. The purpose is to explore the stability of the product by accelerating the chemical or physical changes of the product and provide data for the product packaging, transportation and storage.

Necessary information: The test sample needs 3 batches, according to the commercial packaging, in the temperature of 40 °C ± 2 °C, relative humidity 75% ± 5% for 6 months, the use of constant temperature and humidity box can control 40 °C±2°C, relative humidity 75%±5% for 6 months. The constant temperature and humidity box used at a control temperature of ±2°C and a relative humidity of ±5%, and can detect true temperature and humidity.

Samples were taken at the end of the 1st, 2nd, 3rd, and 6th months of the experiment, and the project was focused on stability. , Compared with the 0-month results, the results are shown in the above table, the data sheet showed no significant changes in the test results.

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Long-term test report of Enzyme Treated Stevia

Batch	Test (mo)	Appearance	Loss on drying (%)	Ashes (%)	Assay (%)	Microbial Limits
20150401	0	White Powder	3.02	0.09	SG:96.16%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	3	White Powder	3.06	0.09	SG: 96.99%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	6	White Powder	3.01	0.09	SG: 96.40%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	9	White Powder	3.04	0.09	SG:96.69%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	12	White Powder	3.02	0.09	SG:96.68%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	18	White Powder	3.01	0.09	SG:96.94%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	24	White Powder	3.05	0.09	SG:96.76%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
20150402	0	White Powder	3.16	0.09	SG:96.47%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	3	White Powder	3.14	0.09	SG:96.66%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	6	White Powder	3.15	0.09	SG:96.54%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	9	White Powder	3.18	0.09	SG:97.27%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	12	White Powder	3.21	0.09	SG:97.73%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	18	White Powder	3.22	0.09	SG:96.99%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	24	White Powder	3.23	0.09	SG:96.81%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
20150403	0	White Powder	3.25	0.09	SG:96.19%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	3	White Powder	3.28	0.09	SG:96.56%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	6	White Powder	3.19	0.09	SG:96.47%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	9	White Powder	3.18	0.09	SG:96.58%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	12	White Powder	3.16	0.09	SG:96.18%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	18	White Powder	3.15	0.09	SG:96.26%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	24	White Powder	3.18	0.09	SG:96.33%	TABC<10 cfu/g Yeast/Mold<10 cfu/g

1, this test is for the test 3 batches, close to the product storage conditions, simulated market packaging. temperature was set at 25 °C ± 2 °C, relative humidity RH60 ± 10% conditions, Placement for a long-term, respectively, in 3,6,9, 12, 18, 24, and 36 months, and samples were taken compared with the results from the 0 month. The results are shown in the above table.

2. According to the test data, at a temperature of 25° C.±2° C. and a humidity of RH 60±10%, the test results of the three batches of the test samples change little, indicating that the quality of the stevioside is very stable within two years.

Appendix 10 TasteRight Refined Enzyme Treated Stevia Stability Report

Test Report on Influence Factors of Refined Enzyme Treated Stevia (Batch: 20150301)

Test Condition	Days	Test Items			
		Appearance	Moisture-absorption Weight gain (%)	Loss on Drying (%)	Assay (%)
	0	White Powder, Odorless or Characteristic	—	3.11	96.09
High temperature (60°C)	5	White Powder, Odorless or Characteristic	—	3.10	96.80
	10	White Powder, Odorless or Characteristic	—	3.08	96.24
High Humidity (RH92.5%)	5	White Powder, Odorless or Characteristic	8.1%	-	96.55
High Humidity (RH75%)	5	White Powder, Odorless or Characteristic	7.8%	—	96.01
Light (4500lx)	5	White Powder, Odorless or Characteristic	—	3.12	96.57
	10	White Powder, Odorless or Characteristic	—	3.10	96.36

1. This test is conducted under more intense conditions than the accelerated test. Its purpose is to explore the inherent stability of the product and to understand the factors affecting its stability as the production process, packaging.

Storage conditions provide scientific basis.

2.1 The batch of samples is placed in a thermostatic drying oven, placed at a temperature of 60°C for 10 days, sampled on the 5th and 10th days, and examined according to the requirements of the stability inspection item.

The results are compared with 0 months and the results are shown in the above table. The test data show that there is no obvious change in the test results, and the test at 40°C is no longer performed.

2.2 The batch of test samples shall be placed in a sealed container of constant humidity and placed at 25 °C under a relative humidity of 90% ± 5% for 10 days. Sampled to be taken on the 5th and 10th days.

The requirements of the inspection project were compared with the 0-month results. The results are shown in the above table. The test data showed that there was no significant change in the content test results. At a relative humidity of 90% ± 5% at 25°C

Placed for 10 days under the conditions, and sampled on the 5th and 10th days, according to the requirements of the stability inspection project requirements, compared with 0 months results, the results are shown in the above table, the test data show that there was no significant change in the content test results.

Therefore, the moisture of the product can be absorbed to saturated status, so no need to inspect the content change results. Even though, we also have done the 10 days observation for your reference only, but not supposed to shown on above table.

3 The batch of test specimens were placed in a light box with a fluorescent lamp under an illumination of 4500 lx, placed for 10 days, and sampled on the 5th and 10th days.

The test is required to be compared with the 0-month results. The results are shown in the above table. The test data show that there is no significant change in the test results.

GRAS Notice – High Purity Glucosylated Steviol Glycosides
 Qufu Shengren Pharmaceutical Co., Ltd., Sunwin Stevia International, & NuNaturals, Inc.

4/25/19

Accelerated test report of Refined Enzyme treated Stevia

Batch	Test (Mo's)	Appearance	Loss on Drying (%)	Ashes (%)	Assay (%)	Microbial limit
20150301	0	White powder	3.11	0.10	SG:96.09%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	1	White powder	3.12	0.10	SG:96.40%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	2	White powder	3.13	0.10	SG:96.22%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	3	White powder	3.12	0.10	SG:96.08%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	6	White powder	3.10	0.10	SG:95.90%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
20150302	0	White powder	3.01	0.10	SG:96.85%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	1	White powder	3.01	0.10	SG:96.45%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	2	White powder	3.02	0.10	SG:96.62%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	3	White powder	3.02	0.10	SG:96.66%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	6	White powder	3.05	0.10	SG:96.47%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
20150303	0	White powder	3.06	0.10	SG:96.10%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	1	White powder	3.06	0.10	SG:96.00%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	2	White powder	3.07	0.10	SG:96.43%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	3	White powder	3.05	0.10	SG:96.16%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	6	White powder	3.01	0.10	SG:96.92%	TABC<10 cfu/g Yeast/Mold<10 cfu/g

1. This test is conducted under more intense conditions than the accelerated test. Its purpose is to explore the inherent stability of the product and to understand the factors affecting its stability as the production process, packaging.

Storage conditions provide scientific basis.

2.1 The batch of samples is placed in a thermostatic drying oven, placed at a temperature of 60°C for 10 days, sampled on the 5th and 10th days, and examined according to the requirements of the stability inspection item.

The results are compared with 0 months and the results are shown in the above table. The test data show that there is no obvious change in the test results, and the test at 40°C is no longer performed.

2.2 The batch of test samples shall be placed in a sealed container of constant humidity and placed at 25 °C under a relative humidity of 90% ± 5% for 10 days. Sampled to be taken on the 5th and 10th days.

The requirements of the inspection project were compared with the 0-month results. The results are shown in the above table. The test data showed that there was no significant change in the content test results. At a relative humidity of 90% ± 5% at 25°C

Placed for 10 days under the conditions, and sampled on the 5th and 10th days, according to the requirements of the stability inspection project requirements, compared with 0 months results, the results are shown in the above table, the test data show that there was no significant change in the content test results. Therefore, the moisture of the product can be absorbed to saturated status, so no need to inspect the content change results.

3 The batch of test specimens were placed in a light box with a fluorescent lamp under an illumination of 4500 lx, placed for 10 days, and sampled on the 5th and 10th days.

The test is required to be compared with the 0-month results. The results are shown in the above table. The test data show that there is no significant change in the test results.

GRAS Notice – High Purity Glucosylated Steviol Glycosides
 Qufu Shengren Pharmaceutical Co., Ltd., Sunwin Stevia International, & NuNaturals, Inc.

4/25/19

Long-term test report of Refined Enzyme treated Stevia

Batch	Test (Mo's)	Appearance	Loss on Drying (%)	Ashes (%)	Assay (%)	Microbial Limit
20150301	0	White powder	3.11	0.10	SG:96.09%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	3	White powder	3.12	0.10	SG: 96.32%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	6	White powder	3.10	0.10	SG: 96.60%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	9	White powder	3.14	0.10	SG:95.77%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	12	White powder	3.16	0.10	SG:96.30%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	18	White powder	3.20	0.10	SG:96.56%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	24	White powder	3.18	0.10	SG:97.07%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
20150302	0	White powder	3.01	0.10	SG:96.85%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	3	White powder	3.02	0.10	SG:96.42%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	6	White powder	3.05	0.10	SG:96.80%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	9	White powder	3.07	0.10	SG:97.23%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	12	White powder	3.08	0.10	SG:96.88%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	18	White powder	3.10	0.10	SG:96.20%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	24	White powder	3.12	0.10	SG:96.27%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
20150303	0	White powder	3.06	0.10	SG:96.10%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	3	White powder	3.05	0.10	SG:96.73%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	6	White powder	3.01	0.10	SG:96.75%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	9	White powder	3.08	0.10	SG:95.92%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	12	White powder	3.10	0.10	SG:95.98%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	18	White powder	3.12	0.10	SG:96.53%	TABC<10 cfu/g Yeast/Mold<10 cfu/g
	24	White powder	3.11	0.10	SG:96.16%	TABC<10 cfu/g Yeast/Mold<10 cfu/g

1, this test is for the test 3 batches, close to the product storage conditions, simulated market packaging. temperature was set at 25 °C ± 2 °C, relative humidity RH60 ± 10% conditions, Placement for a long-term, respectively, in 3,6,9, 12, 18, 24, and 36 months, and samples were taken compared with the results from the 0 month. The results are shown in the above table.

2. According to the test data, at a temperature of 25° C.±2° C. and a humidity of RH 60±10%, the test results of the three batches of the test samples change little, indicating that the quality of the stevioside is very stable within two years.

Appendix 11 Estimated Daily Intake Levels of Steviol Glycosides Preparations

A. Food Uses as Addressed by JECFA, EFSA, FSANZ & Others

JECFA reviewed various estimates of possible daily intake of steviol glycosides (WHO, 2006). Merisant (2008) also listed intended use levels of rebaudioside A for various food applications in their GRAS Notification. Cargill (2008) estimated the possible daily intake of rebaudioside A assuming the use levels would be comparable to aspartame and (Renwick, 2008). BioVittoria (2009) used an exposure estimate of “sucrose equivalents” and the sweetness intensity of Luo Han Guo fruit extract.

B. Estimated Daily Intake

Using different approaches, JECFA (WHO, 2006), Merisant (2008), and Cargill (2008) estimated daily intakes (EDI) ranging from 1.3 – 4.7 mg per kg bw per day.

C. JECFA

- JECFA (WHO, 2006) evaluated information on exposure to steviol glycosides as submitted by Japan, China and the European Commission by the Scientific Committee on Food. They used the Global Environment Monitoring System (GEMS)/Food database to prepare international estimates of exposure to steviol glycosides (as steviol). JECFA assumed that steviol glycosides would replace all dietary sugars at the lowest reported relative sweetness ratio for steviol glycosides and sucrose, which is 200:1.
- The intakes ranged from 1.3 mg per kg bw per day with the African diet to 3.5 mg per kg bw per day with the European diet. Exposures to steviol glycosides assumed full replacement of all dietary sugars in the diets for Japan and the US.
- JECFA concluded that the replacement estimates were highly conservative. They calculated dietary exposure overestimates and would probably be 20 – 30% of these values or 1.0 - 1.5 mg per kg bw per day on a steviol basis or 3.0 – 4.5 mg per kg bw per day for rebaudioside A based on the molecular weight adjustment.

D. EFSA

- On January 13, 2011, EFSA revised its dietary exposure assessment of steviol glycosides. For high consumers, revised exposure estimates to steviol glycosides remain above the established ADI of 4 mg per kg bw (steviol equivalent). For European children aged 1-14 years, revised intake estimates ranged from 1.7 to 16.3 mg per kg bw per day, and for adults, the range was reported to be from 5.6 to 6.8 mg per kg bw per day (EFSA, 2011b).

E. FSANZ

- FSANZ (2008) estimated steviol glycoside dietary intake for adult consumers in New Zealand, assuming a full sugar replacement scenario, resulting in an estimated mean exposure range of 0.3 - 1.0 mg per kg bw per day and the 90th percentile consumer ranged from 0.5 – 1.5 mg per kg bw per day for rebaudioside A. FSANZ concluded that there were no safety concerns for either adults or children.
- In 2009, Cargill applied to FSANZ to increase the maximum usage levels of steviol glycosides in the high-volume food categories with increased usage levels by presenting market share analyses that overestimate actual intake while remaining well below the generally accepted ADI.
- FSANZ (2010) accepted the increased usage levels as requested from Cargill since no public health and safety issues were identified.

F. Merisant

- Merisant (2008) utilized food consumption survey data from 2003-2004 National Health and Nutrition Examination Survey (NHANES) to determine the estimated daily intake from the proposed uses of rebaudioside A.
- On a per user basis, the mean and 90th percentile daily consumption levels of rebaudioside A were estimated as 2.0 and 4.7 mg per kg bw per day, respectively.
- On a steviol equivalent basis, the Merisant estimates calculated to be 0.7 and 1.6 mg per kg bw per day, respectively.
- On December 17, 2008, Merisant (2008) received a “no questions” letter from FDA for the use of rebaudioside A using NHANES food consumption data.

G. Cargill

- Cargill (2008) estimated dietary intake figures for rebaudioside A by assuming that use levels of rebaudioside A would be comparable to those of aspartame uses in the US via post-market surveillance consumption data and published data for consumption of aspartame and other high intensity sweeteners (Renwick, 2008).
- On December 17, 2008, Cargill (2008) received a “no questions” letter from FDA for the use of rebaudioside A using comparative aspartame data.
- On May 13, 2011, FSANZ approved a Cargill application to increase the allowed maximum permitted level (MPL) of steviol glycosides (expressed as steviol equivalents) in ice cream, water based beverages, brewed soft drinks, formulated beverages and flavored soy beverages up to 200 mg per kg and in plain soy beverages up to 100 mg per kg (FSANZ, 2011).

H. BioVittoria

- BioVittoria (2009) used an exposure estimate of “sucrose equivalents” and the sweetness intensity of any particular sweetener based upon data published by Renwick (2008).
- These data resulted in a maximum of 9.9 mg per kg bw per day for any population.
- BioVittoria received a “no questions” letter from FDA for the use of Luo Han Guo fruit extract (SCFE) using Renwick’s “sucrose equivalents” in 2010.

I. Other Publications

- Roberts et al. (2016) suggested that a higher ADI is justified based on metabolic factors to reduce the 100X safety factor. A chemical-specific adjustment factor (CSAF), as defined by the WHO in 2005, is determined by comparative studies in rats and humans.
 - A CSAF that is less than the standard 100X safety factor will result in an increase in the ADI, independent of the NOAEL.
 - The authors determined that using a CSAF can justify an ADI value of 6-16 mg per kg bw per day for steviol glycosides, depending on whether area under the plasma-concentration time curve (AUC) or C_{max} data are used when considering the 1,000 mg per kg bw per day NOAEL (which is equivalent to 400 mg per kg bw per day of steviol) for stevioside reported by Toyoda et al. (1997).

Appendix 12 Studies on Steviol Glycosides Preparations

PART 1. PREPARATIONS THAT ARE PRIMARILY MIXTURES OF STEVIOSIDE & REBAUDIOSIDE A

A. Absorption, Distribution, Metabolism & Excretion (ADME) Studies

1. Animal Studies

- Various animal studies that show stevioside is not readily absorbed from the GI tract:
 - Rats – (Wingard Jr et al., 1980; Nakayama et al., 1986; Koyama et al., 2003b);
 - Chickens – (Geuns et al., 2003b);
 - Hamsters – (Hutapea et al., 1999);
 - Pigs – (Geuns et al., 2003a)
- *In vitro* metabolism studies show stevia glycosides are transformed to steviol which is better absorbed in rats and humans (Geuns, 2003; Koyama et al., 2003b; Gardana et al., 2003; Wang et al., 2004).
- Koyama et al. (2003b) showed steviol can be converted to various glucuronides.
- Excretion of metabolites of stevioside after oral doses has been shown in urine and feces in rats (Sung, 2002) and hamsters (Hutapea et al., 1999).
- Oral doses in pigs led to the detection of metabolites in feces but not in urine (Geuns et al., 2003a).
- Koyama et al. (2003b) published an *in vitro* study where α -glucosylated steviol glycosides were degraded by fecal microflora to steviol glycosides. These glycosides are subsequently hydrolyzed to the aglycone, steviol, demonstrating that the metabolic fate of α -glucosylated steviol glycosides follows that of non-modified steviol glycosides.
- Due to the similarities in metabolic fate, the safety of α -glucosylated steviol glycosides can be established based on studies conducted with non-modified steviol glycosides.
- Since the individual steviol glycosides show similar pharmacokinetics in the rat and humans, the results of toxicology studies on individual steviol glycosides are applicable to the safety of steviol glycosides in general.

2. Human Studies

- Geuns et al. (2006) measured blood, urine, and fecal metabolites in 10 healthy subjects who received 3 doses of 250 mg of purified stevioside (>97%) three times a day for 3 days:
 - Free steviol was detected in feces but not in blood or urine. Steviol glucuronide was detected in blood, urine, and feces. Approximately 76% of the total steviol equivalents dosed were recovered in urine and feces.

- The authors concluded that there was complete conversion of stevioside in the colon to steviol, which was absorbed and rapidly converted to the glucuronide.
- Renwick and Tarka (2008) reviewed studies on microbial hydrolysis of steviol glycosides and concluded that stevioside and rebaudioside A are not absorbed directly but are converted to steviol by gut microbiota in rats and in humans. This hydrolysis occurs more slowly for rebaudioside A than for stevioside.

B. Acute Toxicity Studies

A summary of the acute toxicity of stevioside (96% pure) is presented in Table 12.1.

Table 12.1. Acute Toxicity of Stevioside (Purity 96%) Given Orally to Rodents

SPECIES	SEX	LD ₅₀ (G/KG BW)	REFERENCE
Mouse	Male and Female	>15	Toskulkac et al. (1997)
Mouse	Male	> 2	Medon et al. (1982)
Rat	Male and Female	>15	Toskulkac et al. (1997)
Hamster	Male and Female	>15	Toskulkac et al. (1997)

No lethality was noted within 14 days after the administration, and no clinical signs of toxicity, or morphological or histopathological changes were found, indicating that stevioside is essentially nontoxic in acute oral exposures.

C. Subchronic Toxicity Studies

- Aze et al. (1990) added stevioside at 0, 0.31, 0.62, 1.25, 2.5, 5% to the diets of F344 rats for 13 weeks and reported no adverse effects. The apparent NOAEL was >5% dietary stevioside.
- Mitsuhashi (1976) added up to 7% stevioside to the diets of F344 rats for 3 months and report no adverse effects.
- Akashi and Yokoyama (1975) dosed rats with stevioside up to 2,500 mg per kg bw per day for 3 months and reported no adverse effects.
- The Awney et al. (2011) study revealed toxicity was conducted on rats dosed with stevioside at 15 and 1,500 mg per kg, which resulted in a NOAEL of 15 mg per kg per day. This study is considered to be an outlier in critical reviews by Carakostas (2012) and Waddell (2011) for the following reasons:
 - Insufficient number of animals;
 - Animals were group housed leaving unreliable drinking water quantification;
 - No evidence of fasting before blood collection;
 - No urinalyses;
 - No histopathological confirmation of effects;
 - No organ weight data;
 - No laboratory historical control comparisons;

- Use of tartrate-resistant alkaline phosphatase (TRAP) enzyme which has not been properly vetted for application on toxicological studies;

In summary, the data presented by Awney et al. (2011) are probably not representative of changes due to the subchronic dietary administration of steviol glycosides because of overall inadequate study design and reliance on the findings of the untested enzyme TRAP.

D. Chronic Toxicity Studies

- Toyoda et al. (1997) added stevioside (96.5%) to the diets of F344 rats at 0, 2.5, and 5% for 104 weeks. The authors reported dose-dependent body weight gains decreased in both sexes. Kidney weights were significantly lower in 5% males; ovary, kidney and brain weights were significantly increased in 5% females and there were decreased survival rates in males receiving 5%. However, stevioside was not carcinogenic at any level. The apparent NOAEL was the dietary level of 2.5%.
- Xili et al. (1992) added stevioside (86%) to the diets of F344 rats at 0, 0.2, 0.6, and 1.2% for 3 months and report no adverse effects. The calculated NOAEL was 794 mg per kg bw per day (high dose – 1.2%).
- Yamada et al. (1985) added stevioside to the diets of F344 rats at 0.1, 0.3, and 1.0% with 95.2% steviol (75% stevioside/16% rebaudioside) for 22 months in males and 24 months in females. Differences were note is some parameters; however, the authors concluded that after 2 years of exposure, there were no significant changes that could be attributed to the administration of stevioside and reported no adverse effects. The calculated NOAEL was 550 mg per kg bw per day.
- No treatment-related increase in tumor incidence was seen in any of these studies.

E. Reproductive & Developmental Toxicity Studies

- No effects on pregnancy or developmental parameters were observed in Swiss albino mice administered stevioside or aqueous stevia extract at doses of 500 and 800 mg per kg bw per day for 15 days in female mice (Kumar and Oommen, 2008).
- No effect on fertility or reproductive parameters was seen in a three-generation study in hamsters at doses of 90% stevioside at 0, 500, 1,000, and 2,500 mg per kg bw per day (Yodyingyuad and Bunyawong, 1991). The NOAEL was determined to be 2,500 mg per kg bw per day.
- No effects were observed in rats at doses of 96% stevioside dosed at 0, 0.15, 0.75, or 3% (equivalent to 2,000 mg per kg bw per day). The NOAEL was determined to be 2,000 mg per kg bw per day (Mori et al., 1981).
- No teratogenic effects were observed in an additional rat study that was reviewed by Geuns (2003) where Wistar pregnant female rats were administered stevioside (95.6%) at 0, 250, 500 or 1,000 mg per kg bw per day for 10 days (Usami et al., 1994). The NOAEL was determined to be 1,000 mg per kg bw per day.

- In rat studies, dried stevia leaves were administered at 0.67 g per mL in 2 mL doses twice per day for 60 days (Oliveira-Filho et al., 1989). The only difference due to treatment was seminal vesicle weight, which fell to 60% compared with control. No treatment-related adverse effects were noted.
- In experimental studies in rats, crude stevia leaf extract (5%) was administered to female rats at 0 or 5% for 12 days that were subsequently mated with untreated males for the last 6 days making a total of 18 days exposure for the females (Planas and Kuć, 1968). Fertility was reduced to 21% of fertility in control rats and remained reduced in a 50-60 day recovery. Study report did not discuss histological examinations, weights of organs, blood analysis, urine chemistry, and necropsy.
- The use of *S. rebaudiana* as an oral contraceptive has been reported by indigenous populations in Paraguay (Planas and Kuć, 1968; Schvartaman et al., 1977).
- A developmental study of 90% steviol in hamsters at 0, 250, 500, 750, or 1,000 mg per kg bw per day on days 6-10 of gestation resulted in a significant decrease in body weight gain and increased mortality (1/20, 7/20, and 5/12) at the three highest doses. No dose-dependent teratogenic effects were observed. The NOEL was 250 mg per kg bw per day for both maternal and developmental toxicity (Wasuntarawat et al., 1998).

F. Mutagenicity & Genotoxicity Studies

The following key mutagenicity studies have been conducted on stevia extracts and stevioside and are negative for mutagenic responses:

- Bacterial mutagenicity studies negative for mutagenic response:
 - Matsui et al. (1996)
 - Suttajit et al. (1993)
 - Klongpanichpak et al. (1997)
 - Matsui et al. (1996)
 - Pezzuto et al. (1985)
 - Medon et al. (1982)
- Mouse lymphoma (L5178Y/TK+) study negative for mutagenic response:
 - Oh et al. (1999)
- Chromosome aberration studies negative for mutagenic response:
 - Human lymphocytes – Suttajit et al. (1993)
 - Chinese hamster lung fibroblasts – Nakajima (2000a); Ishidate et al. (1984)
- DNA damage (Comet assay) negative for mutagenic response:
 - Sekihashi et al. (2002)
 - Sasaki et al. (2002)
- Mouse bone marrow/liver micronucleus studies negative for mutagenic response:
 - Oh et al. (1999)

One study was found to be positive and was conducted by Nunes et al. (2007a). The Nunes study revealed toxicity and was conducted on rat liver, brain, and spleen on rats dosed 4 mg per mL steviol glycosides in drinking water (estimated 80 to 500 mg per kg bw per day) for 45 days, which resulted in positive findings in all tissues – notably the liver. This study is considered to be an outlier in critical reviews conducted by Geuns (2007), Williams (2007), and Brusick (2008). These critiques were responded to by the authors (Nunes et al., 2007b; Nunes et al., 2007c). However, the consensus appears to be that Nunes et al. (2007a) used flawed methodology and improperly interpreted data as a positive response.

- In two separate reviews by Carakostas et al. (2008) and Brusick (2008), the recent research on rebaudioside A was summarized and combined with the body of knowledge on stevioside. These authors noted the following:
 - Steviol glycosides, rebaudioside A, and stevioside are not genotoxic *in vitro*.
 - Steviol glycosides, rebaudioside A, and stevioside have not been shown to be genotoxic *in vivo* in well-conducted assays.
 - The Nunes et al. (2007a) study was improperly interpreted as positive.
 - Stevioside is not a carcinogen or cancer promoter in well-conducted rodent chronic bioassays.
- Urban et al. (2013) examined the genotoxicity database on steviol glycosides concluding that the current database of *in vitro* and *in vivo* studies for steviol glycosides is robust and does not indicate that either stevioside or rebaudioside A are genotoxic.

G. Clinical Studies & Other Reports in Humans

In several studies, pharmacological and biochemical effects of crude extracts of stevia leaves and purified steviol glycosides have been investigated. The effects noted included glucose uptake, insulin secretion, and blood pressure (Geuns et al., 2003a). In South America, stevioside is used as a treatment for type 2 diabetes. These effects were key concerns for JECFA. In 2006, JECFA summarized the available clinical studies of stevioside and further studies were recommended (WHO, 2006). Subsequently, several additional studies were conducted, and in 2009, JECFA again reviewed these new studies (WHO, 2009). JECFA’s summaries of the key studies are included in Table 12.2.

Table 12.2: Human Studies with Stevioside Preparations

AUTHOR/ YEAR	SUBSTANCE TESTED	TOTAL DAILY DOSE	POPULATION CHARACTERISTICS	STUDY	NOTED EFFECTS SAFETY PARAMETER RESULTS
Curi et al. (1986)	Aqueous extracts <i>S. rebaudiana</i> leaves	5 g 6 h intervals for 3 days = 15 g/day	16 healthy patients – extract/ 6 healthy patients – arabinose	3-day glucose tolerance in healthy adults	The extract of <i>Stevia rebaudiana</i> increased glucose tolerance. The extract decreased plasma glucose levels during the test and

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AUTHOR/ YEAR	SUBSTANCE TESTED	TOTAL DAILY DOSE	POPULATION CHARACTERISTICS	STUDY	NOTED EFFECTS SAFETY PARAMETER RESULTS
					after overnight fasting in all volunteers.
Chan et al. (2000)	Stevioside (purity not stated)	750 mg (11 mg per kg bw/day)	60 hypertensive Chinese men and woman aged 28-75) + 46 patients were given placebo.	Multicenter randomized, double-blind, placebo-controlled for 12 months	3 months: mean systolic and diastolic BP decreased and continued through the 12 months. Minor side effects occurred with 2 test group and 1 placebo group patient withdrawing. Other side effects were minor and resolved.
Hsieh et al. (2003)	Stevioside (purity not stated)	1,500 mg (21 mg/kg bw/day)	85 hypertensive Chinese men and woman aged 20-75) + 89 patients were given placebo.	Multicenter randomized, double-blind, placebo-controlled for 24 months	Mean systolic and diastolic blood pressures were decreased commencing from about 1 week after the start of treatment. At 2 years test group patients had ↓ in incidence of left ventricular hypertrophy. 3 patients withdrew. Other side effects were minor and resolved.
Anonymous (2004a)	Steviol extract: (~73 % stevioside ~24% Reb A)	100 mg (3.3 mg/kg bw/day)	48 hyperlipidemic volunteers (24/24)	Randomized, double-blind, placebo-controlled for 3 months	Analyses of serum concentrations of triglycerides, liver-derived enzymes, and glucose indicated no adverse effects. 3 patients withdrew. No adverse side effects were reported.
Anonymous (2004b)	Steviol extract: (~73% stevioside ~24% Reb A)	3.25, 7.5 and 5 mg/kg bw/day	12 patients per test group	Randomized, double-blind, placebo-controlled for 30 days	No adverse responses in blood and urine biochemical parameters
Gregersen et al. (2004)	Stevioside - 91% + 9% other stevia glycosides	Single dose 1 g stevioside or 1 g starch	12 patients with type 2 diabetes total	Acute paired cross-over study	↓18% glucose concentrations: Systolic and diastolic blood pressure unchanged. No adverse effects
Temme et al. (2004)	Stevioside 97%	750 mg/kg bw/day (288 mg/kg bw steviol)	4 male 5 female healthy patients	Short term study – 3 days	Blood chemistry, blood pressure and urinalyses were unremarkable
Barriocanal et al. (2006)	Stevioside – 64.5% + 18.9% Reb A	750 mg/kg bw/day	Type 1 (n=8) + Type 2 (n=15) diabetics + non-diabetics (n=15) + matching controls - placebo	Double-blind, placebo-controlled trial study for 3 months	Blood chemistry, glycated hemoglobin (HbA1c), blood pressure and urinalyses were unremarkable. No adverse effects

AUTHOR/ YEAR	SUBSTANCE TESTED	TOTAL DAILY DOSE	POPULATION CHARACTERISTICS	STUDY	NOTED EFFECTS SAFETY PARAMETER RESULTS
Barriocanal et al. (2008)	Stevioside - >92%	250 mg/kg bw/day	Type 1, Type 2 , placebo controls	Randomized, double-blind, placebo-controlled for 3 months	No changes in systolic BP, diastolic BP, glucose, or glycated hemoglobin from baseline. No adverse effects
Ferri et al. (2006)	Stevioside (purity not stated)	3.75, (7 weeks), 7.5 (11 weeks), 15 (6 weeks) + placebo (24 weeks mg/kg bw/day	Patients with mild hypertension	Randomized 24 week study	No changes in systolic BP, diastolic BP. No adverse effects
Silva et al. (2006)	Stevioside: 70%	Equivalent to 1.04 mg steviol/kg bw/day + placebo	49 Mild hyperlipidemic patients: Stevioside group (n=24) placebo controls (n=25) Age: 20-70	Placebo-controlled double-blind trial for 90 days	No effects of treatment on ALT, AST, or GGT were found. No relevant adverse effects were noted.
Jeppesen et al. (2006)	Stevioside (purity not stated)	1,500 mg/kg bw/day or maize starch placebo	55 patients with Type 2 diabetes:	Randomized, double blinded, placebo-controlled study	No effects on the HbA1c, fasting blood glucose levels, lipids or blood pressure

PART 2. PREPARATIONS THAT ARE PRIMARILY REBAUDIOSIDE A

A. Absorption, Distribution, Metabolism & Excretion (ADME) Studies

1. Animal Studies

Studies investigating the ADME of extracts from stevia are available on stevioside, rebaudioside A, and other steviol glycosides. Data evaluating the absorption and fate of these extracts from various animal species and humans indicate that one can extrapolate these results from rats to humans.

- Studies investigating the hydrolysis of steviol glycosides by intestinal microflora have demonstrated that both stevioside and rebaudioside A are hydrolyzed to steviol following *in vitro* incubation with various cecal microflora (Wingard Jr et al., 1980; Hutapea et al., 1997; Gardana et al., 2003; Geuns et al., 2003a).
- *In vitro* hydrolysis of rebaudioside A to steviol was found to be slower than that of stevioside (Koyama et al., 2003a).
 - The major pathway for rebaudioside A is conversion to stevioside with a minor pathway of conversion to rebaudioside B prior to being ultimately converted to steviol. Stevioside is further converted to steviolbioside, steviolmonosides, and finally steviol, with glucose being released with each subsequent hydrolysis.

- Roberts and Renwick (2008) identified free steviol (82 to 86%), steviol, glucuronide (10 to 12%), and two unidentified metabolites (5-6%) in rat plasma following treatment with either stevioside or rebaudioside A eight hours post-oral administration. Steviol T_{max} for plasma was noted within 30 minutes of oral administration as opposed to rebaudioside A with a T_{max} of 2 to 8 hours.
 - Following rebaudioside A treatment, significant amounts of unchanged rebaudioside A (29% in males and 19% in females) and stevioside (3% in males and 4% in females) were excreted in the feces.
 - Urinary excretion accounted for less than 2% of the administered dose.
 - Steviol was the predominant component found in plasma samples after oral administration of rebaudioside A, stevioside, and steviol in rats. The majority of all samples were found to be excreted rapidly---primarily in the feces---within 48 hours.
 - The predominant compound detected in the bile was steviol glucuronide, while the prominent material in the intestine was steviol.
 - The authors concluded that the overall data on toxicokinetics and metabolism indicate that rebaudioside A and stevioside are handled in an almost identical manner in the rat after oral dosing.
- Wheeler et al. (2008) assessed the comparative pharmacokinetics of steviol and steviol glucuronide following single oral doses of rebaudioside A and stevioside.
 - Following administration of rebaudioside A or stevioside, steviol glucuronide appeared in the plasma of all subjects, with median T_{max} values of 12.0 and 8.00 hours post-dose, respectively.
 - Administration of rebaudioside A resulted in a significantly (~22%) lower steviol glucuronide geometric mean C_{max} value (1,472 ng per mL) than administration of stevioside (1,886 ng per mL). The geometric mean AUC_{0-t} value for steviol glucuronide after administration of rebaudioside A (30,788 ng*hr per mL) was approximately 10% lower than after administration of stevioside (34,090 ng*hr per mL).
 - The authors concluded that rebaudioside A and stevioside underwent similar metabolic and elimination pathways in humans, with steviol glucuronide excreted primarily in the urine and steviol in the feces.
 - No safety concerns were noted as determined by reporting of adverse events, laboratory assessments of safety, or vital signs.
- Slotter (2008a) examined the potential of rebaudioside A toxicity in rats up to 2,000 mg per kg bw per day
 - Low levels of rebaudioside A were detected in peripheral blood of rats post administration of 2,000 mg per kg bw per day.
 - Mean plasma concentrations of rebaudioside A of 0.6 µg per mL in plasma resulting in an estimated absorbed dose of 0.02% based on amounts calculated from urine collection.

- Mean fecal rebaudioside A and measured hydrolysis products, expressed as Total Rebaudioside A Equivalents, compared to daily administered dose results in an estimated dose recovery of approximately 84%.

B. Subchronic Toxicity Studies

- Curry and Roberts (2008) added up to 100,000 ppm of rebaudioside A (97%) to the diets of Wistar rats for 13 weeks and reported no treatment-related adverse effects. Hence, the NOAEL was reported to be 9,938 mg per kg males and 11,728 mg per kg females – the highest level of treatment.
- Rebaudioside A (99.25%) was added to the diets of CRL:CD(SD) rats for 90 days at target doses of 500, 1,000, and 2,000 mg per kg bw per day with no treatment-related effects. The NOAEL was determined to be $\geq 2,000$ mg per kg (Eapen, 2007; Nikiforov and Eapen, 2008).
- Eapen (2008) added rebaudioside A (97.5%) to the diets of Beagle dogs for 6 months at target doses of 500, 1,000, and 2,000 mg per kg bw per day and reported no adverse effects. The NOAEL was determined to be $> 2,000$ mg per kg bw per day.
- The oral administration of fermentative Reb A to Sprague-Dawley rats for 91 days did not lead to any adverse effects at consumption levels up to 2,057 mg per kg bw per day for males and 2,023 mg per kg bw per day for females, which were concluded to be the NOAELs (Rumelhard et al., 2016).

C. Mutagenicity & Genotoxicity Studies

- *In vitro* and *in vivo* genotoxicity assays covering mutation, chromosome damage, and deoxyribonucleic acid (DNA) strand breakage consistently and uniformly revealed negative results for rebaudioside A.
- Evaluation of fermentation-derived rebaudioside A demonstrated a similar safety profile to plant-derived rebaudioside A (Rumelhard et al., 2016).

The following key mutagenicity studies have been conducted on rebaudioside A and are negative for mutagenic responses :

- Bacterial mutagenicity studies negative for mutagenic response:
 - Wagner and Van Dyke (2006)
 - Williams and Burdock (2009)
 - Rumelhard et al. (2016)
- Mouse lymphoma (L5178Y/TK+/) studies negative for mutagenic response:
 - Clarke (2006)
 - Williams and Burdock (2009)
 - Human lymphocyte study negative for mutagenic response: Rumelhard et al. (2016)
- Chromosome aberration studies negative for mutagenic response:
 - Human lymphocytes – Williams and Burdock (2009)

- Chinese hamster lung fibroblasts – Nakajima (2000a)
- Mouse micronucleus studies negative for mutagenic response:
 - Krsmanovic and Huston (2006)
 - Williams and Burdock (2009)
 - Nakajima (2000b) (BDF1 mouse bone marrow)
 - Unscheduled DNA synthesis (UDS) study negative for mutagenic response - Williams and Burdock (2009)
- Bacterial forward mutation study negative for mutagenic response – Pezzuto et al. (1985)

D. Reproductive & Developmental Studies

- Curry et al. (2008) conducted a 2-generation reproductive toxicity study on rebaudioside A administered in the diet at 7,500, 12,500 and 25,000 ppm in Han Wistar rats. There were no signs of toxicity or adverse effects on body weights, body weight gain, or food consumption. Rebaudioside A did not affect reproductive performance parameters including mating performance, fertility, gestation lengths, estrous cycles, or sperm motility, concentration, or morphology in either the F₀ or F₁ generations. The NOAEL for reproductive effects was 25,000 ppm, and the NOAEL for the survival, development, and general condition of the offspring also was considered to be 25,000 ppm, or 2,048 to 2,273 mg per kg bw per day (the highest dose tested).
- An unpublished study on rebaudioside A was conducted on four groups of male and female Crl:CD(SD) rats (30 per sex per group) were fed either basal diet or the diet containing rebaudioside A (purity 95.7%) for at least 70 consecutive days prior to mating (Sloter, 2008a). The test diet was offered to the offspring selected to become the F₁ generation following weaning (beginning on postnatal day 21). The F₀ and F₁ males continued to receive rebaudioside A throughout mating, gestation, and lactation until day of euthanasia. Both for parental systemic and reproductive toxicity, the NOAEL was ≥2,000 mg per kg bw per day (highest dose administered).
- In another unpublished study, the embryo/fetal developmental toxicity effects were studied in rats (Sloter, 2008b) on rebaudioside A administered via gavage. The NOAEL for maternal and embryo/fetal development was determined to be >2,000 mg per kg bw per day.

E. Clinical Studies on Rebaudioside A

A summary of the clinical studies conducted on rebaudioside A is presented in Table 12.3.

Table 12.3. Human Studies with Rebaudioside A Preparations

AUTHOR/ YEAR	SUBSTANCE TESTED	TOTAL DAILY DOSE	POPULATION CHARACTERISTICS	STUDY	NOTED EFFECTS SAFETY PARAMETER RESULTS
Maki et al. (2008a)	Rebaudioside A (97%)	Reb A: 1,000 mg Placebo: 0	100 patients with normal and low-normal systolic blood pressure (SBP) and diastolic blood pressure (DBP)	Randomized, double-blind, placebo-controlled trial for 4 weeks	The extract of <i>Stevia rebaudiana</i> increased glucose tolerance. The extract decreased plasma glucose levels during the test and after overnight fasting in all volunteers.
Maki et al. (2008b)	Rebaudioside A (97%)	Reb A: 1,000 mg (n=60) Placebo: 0 (n=62) Age: 33-75	Men and women with Type 2 diabetes	Randomized, double-blind, placebo-controlled trial for 16 weeks	No treatment related changes in blood pressure, body weight, and fasting lipids were noted. Rebaudioside A was well-tolerated, and records of hypoglycemic episodes showed no excess versus placebo

F. Safety of Rebaudioside A

There have been a significant number of studies regarding the safety and toxicity of rebaudioside A:

- GRAS notifications submitted to FDA by:
 - GRN 252: Merisant (2008) conducted studies that augmented genotoxicity data in three systems recognized by FDA as good predictors of carcinogenic potential. Two of these assays were conducted in mouse systems.
 - GRN 253: Cargill (2008) conducted studies that provided significant insight into the pharmacokinetics of rebaudioside A, while demonstrating clinical safety of rebaudioside A regarding lack of effects on blood pressure and glucose metabolism that could result from doses expected from use in food.
- JECFA concluded that all naturally occurring steviol glycosides are deemed to be safe as long as there is a combined purity of 95% and determined the ADI of the steviol glycosides applied to rebaudioside A because the pharmacokinetics are virtually the same (FAO, 2017).
- Carakostas et al. (2008) summarized the Cargill research program findings on rebaudioside A:

- Steviol glycosides, rebaudioside A, and stevioside are not genotoxic *in vitro*.
- In well-conducted *in vivo* assays, steviol glycosides, rebaudioside A, and stevioside have not been found to be genotoxic.
- A report indicating that stevioside produces DNA breakage *in vivo* appears to be flawed (Nunes et al., 2007a) and was improperly interpreted as a positive response.
- Steviol genotoxicity in mammalian cells is limited to *in vitro* tests that may be affected by excessive concentrations of the compound.
- The primary evidence for steviol genotoxicity is derived from very specific bacterial tests or purified plasmid DNA that lack DNA repair capabilities.
- Stevioside is not a carcinogen or cancer promoter in well-conducted rodent chronic bioassays.
- While studies with rebaudioside A indicated slight gastrointestinal (GI) absorption of the glycoside per se, the predominant metabolic pathway is comparable to that of stevioside and the use of the ADI established by JECFA, which was determined on studies employing stevioside as the main component, can be used as the ADI for rebaudioside A.
- The dietary levels expected from consumption of rebaudioside A as a total replacement of sugar (Renwick, 2008) are less than the ADI and, therefore, there is no safety concern for consumers.
- JECFA has evaluated the use of steviol glycosides in foods and agrees that, at the present time, the ADI for steviol glycosides of adequate purity, as defined by JECFA specifications, has been properly determined to be 4 mg per kg bw per person as steviol equivalents, which corresponds to 12 mg per kg bw per day for rebaudioside A, on a dry weight basis. Therefore, the JECFA-derived ADI was adopted as a safe exposure for rebaudioside A and the corresponding food uses meeting the specifications within the limits determined by this esteemed international body of food safety experts can be considered to be GRAS.
- Williams and Burdock (2009) reviewed 3 *in vitro* and 2 *in vivo* genotoxicity and mutagenicity studies on rebaudioside A conducted according to OECD guidelines and found the studies revealed that rebaudioside A is:
 - Non-mutagenic in an Ames test using *Salmonella typhimurium* and *Escherichia coli*
 - Non-mutagenic in a chromosomal aberration test using Chinese hamster V79 cells
 - Non-mutagenic in a mouse lymphoma assay using L5178Y+/- cells
 - Non-mutagenic a bone marrow micronucleus test in mice at doses up 750 mg per kg bw
 - Non-mutagenic in an unscheduled DNA synthesis test in rats at 2,000 mg per kg bw.
 - The authors note that these studies provide additional evidence that rebaudioside A is not genotoxic at the doses tested and further support the GRAS determination of rebaudioside A.

PART 3. STUDIES ON PRINCIPAL METABOLITE: STEVIOL

A. Acute Toxicity Studies

- Toskulkac et al. (1997) administered single doses of steviol (90%) to various animals as follows:
 - Rat, oral LD₅₀ >15 g per kg
 - Hamster, oral LD₅₀ 5.2 g per kg bw in males and 6.1 g per kg bw in females
 - Histopathological examination of the kidneys revealed severe degeneration of the proximal tubular cells, and these structural alterations were correlated with increased serum blood urea nitrogen and creatinine. The authors concluded that the cause of death was acute renal failure.

B. Developmental Toxicity Studies

- Wasuntarawat et al. (1998) dosed groups of pregnant golden hamsters steviol (90%) at 0, 250, 500, or 1,000 mg per kg bw per day by gavage in corn oil on days 6 -10 of gestation (1,000 mg group, n=12).
 - A significant decrease in body weight gain and increased mortality (1/20, 7/20, and 5/12) were observed at the three highest doses.
 - The number of live fetuses per litter and mean fetal weight decreased in parallel.
 - No dose-dependent teratogenic effects were seen.
 - NOEL for both maternal and developmental toxicity was 250 mg per kg bw per day.

C. Mutagenicity & Genotoxicity Studies

The following key mutagenicity studies have been conducted on steviol and are negative for mutagenic responses:

- Bacterial mutagenicity studies negative for mutagenic response:
 - Klongpanichpak et al. (1997)
 - Procinska et al. (1991)
 - Compadre et al. (1988)
- Chromosome aberration studies negative for mutagenic response:
 - Chinese hamster lung fibroblasts – Matsui et al. (1996)
- DNA damage (Comet assay)
 - Sekihashi et al. (2002)
- Mouse bone marrow/liver micronucleus studies negative for mutagenic response:
 - Oh et al. (1999)
- Micronucleus studies negative for mutagenic response:
 - Temcharoen et al. (2000) (rat)
 - Temcharoen et al. (2000) (mouse)
 - Matsui et al. (1996) (mouse)

- Temcharoen et al. (2000) (hamster)

The following key mutagenicity studies have been conducted on steviol and are positive or equivocal for mutagenic responses:

- Bacterial mutagenicity studies positive for mutagenic response:
 - Matsui et al. (1996) – Steviol was equivocal for mutagenicity. Steviol was weakly positive in Umu chromotest, either with or without metabolic activation. Steviol negative in reverse mutation and other bacterial assays even in presence of S9 activation
 - Terai et al. (2002) – Steviol found to be mutagenic in Aroclor-induced rat liver S9 fraction.
 - Temcharoen et al. (2000) – Mutagenic effects of steviol and/or metabolites found in *S. typhimurium* TM677 by tranversions, transitions, duplications, and deletions at the guanine phosphoribosyltransferase (gpt) gene.
 - Pezzuto et al. (1985) – Mutagenicity was dependent on pretreatment of rats with Aroclor and NADPH addition, as unmetabolized steviol was inactive. None of the other metabolites tested was mutagenic.
 - Compadre et al. (1988) – Mass spectral analysis of steviol and analogues under conditions known to produce a mutagenic response. 15-oxo-steviol, a product of the metabolite, 15-alpha-hydroxysteviol was found to be direct-acting mutagen.
- Chinese hamster lung fibroblast study positive for mutagenic response:
 - Matsui et al. (1996) – Gene mutations found in Chinese hamster lung fibroblasts after metabolic activation of steviol. In hamsters, several metabolites of stevioside found that have not been found in rats or humans. Therefore, experimental relevance should be questioned when hamsters are used.

Each of the positive mutagenicity studies noted above had special circumstances or slightly different procedures and were collectively not believed to present sufficient toxicological concern as determined by JECFA (WHO, 2006).

D. Endocrine Disruption Studies

- Shannon et al. (2016) investigated the endocrine disrupting potential of stevioside, rebaudioside A, and steviol in a series of *in vitro* bioassays and found that steviol:
 - antagonizes progesterone nuclear receptor transcriptional activity
 - increases progesterone production
 - induces an agonistic response on the progesterone receptor of sperm cells (Catsper)

The authors conclude that steviol might not qualify as a safer alternative to sugar or synthetic sweeteners. However, one must consider the fact that it is difficult to translate *in vitro* concentrations to local concentrations *in vivo* at the receptor level and no adverse effects have been noted in any reproductive studies.

Appendix 13 Summary of the Regulatory History of Steviol Glycosides

A. History of Traditional Medicinal and Human Food Use

- Stevia use as a sweetener and in traditional medicine by the Guarani tribes can be traced back for centuries (Esen, 2016; Gerwig et al., 2016; Brusick, 2008; Brandle et al., 1998).
- Commonly used to treat Type 2 diabetes in South America (Hawke, 2003). Doses in the range of 1 gram per person per day or more were reported to be necessary for therapeutic effects (Gregersen et al., 2004).
- Japan and Brazil approved stevia as a food additive in the 1980s (Raintree, 2012). Lester (1999) reported that 40% of the artificial sweetener market in Japan was stevia based.
- Use of steviol glycosides as a dietary supplement is presently permitted in the US, Canada, Australia, and New Zealand, and as a natural health product in Canada.
- In 2005, it was estimated that sales of stevia in the US reached \$45 million (Newsday, 2006).
- In 2010, Zenith International estimated stevia sales of 3,500 metric tons, which represents a 27% increase over 2009 figures. The market value is estimated to have increased to \$285 million (Zenith, 2011).
- In 2013, worldwide sales of stevia was reported at 4,100 tons – representing a 6.5% increase over 2011 figures with an overall market value of \$304 million (Zenith, 2013).
- In October 2014, it was reported that worldwide stevia sales increased 14% to 4,670 tons, with a market value of \$336 million. It has been projected that the total market for stevia in 2017 would be 7,150 tons with an associated market value of \$578 million (Zenith, 2014).
- NewHope360 reported that the global market for stevia in 2014 was \$347 million, and that is expected to increase to \$565.2 million by 2020. In addition, consumption is expected to increase from 2014 levels of 5,100.6 tons to 8,506.9 tons by 2020 (NewHope360, 2015).
- Nutritional Outlook reported that Mintel data indicated a 48% increase in stevia-containing products over the last five years (Decker and Prince, 2018).

B. Summary of Regulatory History of Enzyme Modified Steviol Glycosides

1. U.S. Regulatory History

To date, FDA has issued 56 “no questions” letters on GRAS Notices on rebaudioside A, rebaudioside D, rebaudioside M, or steviol glycosides, including those undergoing enzyme treatment (FDA, 2019).

In addition, the Flavor and Extract Manufacturers Association (FEMA) has included several steviol glycosides preparations that are used to formulate flavors on their GRAS lists as shown in Table 13.1.

Table 13.1. FEMA GRAS Status for Steviol Glycoside Preparations

STEVIOL GLYCOSIDES PREPARATION	FEMA NUMBER	REFERENCE
Rebaudioside A	4601	Smith et al. (2009)
Rebaudioside C; dulcoside B	4720	Leffingwell (2011)
Glucosyl steviol glycosides; enzymatically modified stevia extract	4728	Leffingwell and Leffingwell (2014); Marnett et al. (2013)
Stevioside	4763	Leffingwell and Leffingwell (2014); Marnett et al. (2013)
Steviol glycoside extract, <i>Stevia rebaudiana</i> , Rebaudioside A 60%	4771	Marnett et al. (2013)
Steviol glycoside extract, <i>Stevia rebaudiana</i> , Rebaudioside A 80%	4772	Marnett et al. (2013)
Steviol glycoside extract, <i>Stevia rebaudiana</i> , Rebaudioside C 30%	4796	Cohen et al. (2015a); Cohen et al. (2015b)
Steviol glycoside extract, <i>Stevia rebaudiana</i> , Rebaudioside A 22%	4805	Cohen et al. (2015a); Cohen et al. (2015b)
Steviol glycoside extract, <i>Stevia rebaudiana</i> Rebaudioside C 22%	4806	Cohen et al. (2015a); Cohen et al. (2015b)
Glucosylated stevia extract Steviol glycosides 80%	4845	Cohen et al. (2017)
Enzyme modified stevia, stevioside 20%	4876	Cohen et al. (2017)

2. Canadian Regulatory History

- On September 18, 2009, the Natural Health Products Directorate, Health Canada (Health Canada, 2009) adopted and revised the maximum limit for steviol glycosides in Natural Health products (NHP) to be in accordance with the full acceptable daily intake (ADI) of 4 mg steviol per kg bw established by JECFA (WHO, 2008).
 - As a Medicinal Ingredient: The maximum daily limit without cautionary labelling and additional safety evidence was set at 4 mg per kg bw per day expressed as steviol content. This limit is equivalent to 10 mg per kg bw per day (i.e. ~ 710 mg per day for an adult) for stevioside or mixed steviol glycosides, 12 mg per kg bw per day (i.e. ~ 850 mg per day for an adult) for rebaudioside A, or 50 mg per kg bw per day (i.e. ~ 3,550 mg per day for an adult) of stevia leaf.
 - As a Non-Medicinal Ingredient: As a sweetener or flavor enhancer, the quantity used should be according to conditions of cGMP and should not exceed the amount required to accomplish the purpose for which that non-medicinal ingredient is

permitted to be added. As a non-medicinal ingredient, it should not exceed 4 mg per kg bw per day expressed as steviol content.

- On November 30, 2012, Health Canada published its final clearance for use of steviol glycosides as a sweetener in foods (Health Canada, 2012).
- In March 2014, Health Canada updated the List of Permitted Sweeteners (Lists of Permitted Food Additives) to include steviol glycosides in applications as a table-top sweetener and as an ingredient in a variety of foods, beverages, baked goods, meal replacement bars, condiments, and confectionary and gums (Health Canada, 2014).
- On January 15, 2016, Health Canada approved the use of rebaudioside M for use as a high-intensity sweetener under the same conditions as the previously approved steviol glycosides (Health Canada, 2016).
- Health Canada (2017a) also modified the List of Permitted Sweeteners to include “all the steviol glycosides in the *Stevia rebaudiana* Bertoni plant (stevia plant).”
- On August 30, 2017, Health Canada’s Food Directorate updated its List of Permitted Sweeteners to allow for the use of steviol glycosides as a sweetener in ‘unstandardized snack bars,’ including granola bars, cereal bars, fiber bars, and protein isolate-based bars (Health Canada, 2017b).
- On August 27, 2018, Health Canada’s Food Directorate updated its List of Permitted Sweeteners to provide stakeholders with further information on the Lists of Permitted Food Additives as well as guidance on how to interpret and use these lists (Health Canada, 2018).

3. European Regulatory History

- The Joint Expert Committee on Food Additives (JECFA) reviewed steviol glycosides at its 51st, 63rd, 68th and 73rd meetings and published its original review in 2000 (WHO, 2000).
- In 2006, JECFA established a temporary ADI (acceptable daily intake) of 0-2 mg per kg (on a steviol basis) at its 63rd meeting (WHO, 2006).
- In 2007, JECFA finalized food grade specifications (FAO, 2007b), although they were subsequently updated in 2008 (FAO, 2008) and 2010 (FAO, 2010).
- In 2009, at the 69th meeting, the temporary status of the ADI was removed, and the ADI was raised to 0-4 mg per kg bw per day (on a steviol basis) as a result of the JECFA review of more recently completed clinical studies with steviol glycosides (WHO, 2008). In 2009, JECFA published a final monograph addendum on steviol glycosides (WHO, 2009).
- In 2009, several countries and the Calorie Control Council, submitted a request to the Codex Committee on Food Additives to modify the JECFA specifications for steviol glycosides to include rebaudioside D and rebaudioside F as specifically named acceptable glycosides that would be considered as part of the minimum 95% steviol glycosides composition (CCFA, 2009). The proposal was discussed at the June, 2010 JECFA Meeting (FAO/WHO, 2009), and JECFA subsequently took final action in approving the modified steviol glycosides specifications to include rebaudioside D and rebaudioside F (FAO, 2010).

- In 2008, Switzerland’s Federal Office for Public Health approved the use of stevia as a sweetener citing the favorable actions of JECFA (Switzerland Federal Office of Public Health, 2008).
- In 2009, France published its approval for the food uses of rebaudioside A with a purity of 97% (AFSSA, 2009a; AFSSA, 2009b).
- In June 2008, the European Commission requested for EFSA to deliver a scientific opinion on the safety of steviol glycosides as a sweetener for use in the food categories specified in the dossiers from three petitioners.
 - EFSA reexamined the safety of steviol glycosides (EFSA, 2010), the EFSA Panel established an ADI for steviol glycosides, expressed as steviol equivalents, of 4 mg per bw per day, which is similar to JECFA’s determination.
 - On May 25, 2011, EFSA published the daily dietary intake for use of rebaudioside A as a flavoring substance in a variety of foods would be less than the ADI for steviol glycosides (EFSA, 2011a).
 - In 2014, EFSA evaluated extending the use of steviol glycosides as ingredients in food categories to include coffee, tea, and herbal and fruit infusions (assessed at 10 mg per L steviol glycosides) (EFSA, 2014).
 - In 2015, EFSA revised exposure estimates based on the EFSA Comprehensive European Food Consumption Database and the proposed extension of use for tea beverages and instant coffee and cappuccino products up to 29 mg per L of steviol equivalents, rather than 10 mg per L, as assessed in the previous 2014 EFSA opinion. EFSA noted that the mean exposure estimates remain below the ADI of 4 mg per kg bw per day for all population groups, with the exception of toddlers (in one country) at the upper range of the high-level exposure estimates (95th percentile: 4.3 mg per kg bw per day), which remains above the ADI. EFSA concluded that dietary exposure to steviol glycosides (E 960) is similar to the exposure estimated in 2014 and therefore does not change the outcome of the safety assessment (EFSA, 2015).
- On December 2, 2011 the EU approved steviol glycosides use as food additives (EU, 2011) based upon agreement between the JECFA and EFSA that steviol glycosides are safe for all populations to consume and are a suitable sweetening option for diabetics.
- On November 3, 2016 the EU food additives regulation 231/2012 was amended to remove the previous requirement for stevia blends to contain at least 75% Reb A or stevioside.
- On October 13, 2016 the EU updated regulation EU 2016/1814 to permit the following steviol glycosides in stevia blends: stevioside, rebaudiosides A, B, C, D, E, F and M, steviolbioside, rubusoside, and dulcoside (Searby, 2016).
- On January 31, 2018, the EFSA Panel of Food Additives and Nutrient Sources reviewed an application for glucosylated steviol glycoside preparations for use as a new food additive. The Panel concluded that the data supplied by the applicant were “insufficient to assess the safety” of the preparation. No safety concerns were raised by the EFSA Panel; however, their decision was based on the “limited” data provided in the dossier submitted by the applicant (EFSA, 2018).

4. Asian Regulatory History

- In May 2010, Hong Kong amended its food regulations to allow the use of steviol glycosides as a permitted sweetener in foods based upon the detailed safety evaluation and favorable findings as reported by JECFA (Hong Kong Centre for Food Safety, 2010).
- In July 2011, the Codex Alimentarius Commission adopted proposed maximum use levels for steviol glycosides in all major food and beverage categories which resulted in steviol glycoside approvals in Vietnam, the Philippines, Malaysia, Singapore and Thailand (Whitehead, 2013).
- The International Alliance of Dietary/Food Supplement Associations (IADSA) reported that the Codex Alimentarius Commission agreed to adopt the use of steviol glycosides for addition to chewable food supplements (NewHope360, 2011).
- On September 20, 2012 the Food Safety and Standards Authority of India (FSSAI) approved the use of steviol glycosides as a non-nutritive sweetener in a variety of foods using specifications and purity established by JECFA (FSSAI, 2012).
- Since December 10, 2012 over thirty registrations have been granted by FDA Philippines to stand-alone steviol glycosides sweeteners or foods containing steviol glycosides as ingredients (Philippines, 2014).
- Steviol glycosides are also listed under INS number 960 in the Food Additives Permitted Under the Singapore Food Regulations document prepared by the Agri-Food & Veterinary Authority (AVA) of Singapore (AVA, 2014)

5. Australia and New Zealand Regulation History

- In 2008, the Food Standards Australia New Zealand (FSANZ) completed its evaluation of an application for use of steviol glycosides in foods and recommended that the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council) amend the Australia New Zealand Food Standards Code to allow the use of steviol glycosides in food (FSANZ, 2008).
- On May 13, 2011, FSANZ approved an increase in the maximum permitted level (MPL) of steviol glycosides (expressed as steviol equivalents) in ice cream, water based beverages, brewed soft drinks, formulated beverages, and flavored soy beverages up to 200 mg per kg, and in plain soy beverages up to 100 mg per kg (FSANZ, 2011).
- In 2015, FSANZ concluded that the use of Reb M does not pose any “public health and safety issues” (FSANZ, 2015).
- On January 14, 2016, Reb M was approved for use “as a food additive in accordance with the current permissions for steviol glycosides” (FSANZ, 2016a).
- In 2016, FSANZ called for submissions on permitting all minor steviol glycosides extracted from stevia leaf to be included in the definition of steviol glycosides in the Food Standards Code, noting that “[no] evidence was found to suggest that the proposed changes pose any public health and safety concerns” (FSANZ, 2016b).

GRAS Notice – High Purity Glucosylated Steviol Glycosides
Qufu Shengren Pharmaceutical Co., Ltd., Sunwin Stevia International, & NuNaturals, Inc.

4/25/19

- On February 8, 2017 FSANZ approved a draft variation of the definition of steviol glycosides to include all steviol glycosides present in the *Stevia rebaudiana* leaf (FSANZ, 2017).

6. South Africa

- On September 10, 2012, the South African Department of Health promulgated a new sweetener regulation: Regulation R733 (Regulations Relating to the Use of Sweeteners in Foodstuffs), allowed for the use of extracts of *stevia rebaudiana*, in composition and quantities in line with Codex standards, in food and beverages. Steviol glycosides can be used to a maximum level of 330 mg per kg (Food Stuff South Africa, 2012).

Appendix 14 Summary of Published Safety Reviews

A. Summary of JECFA Reviews

- 51st Meeting (WHO, 2000) – stevioside evaluation determined that there was insufficient and inconsistent information on the stevioside or steviol. No human metabolism data or mutagenicity data were available. JECFA determined that the ADI could not be determined without further data.
- 63rd Meeting (WHO, 2006) – More data were submitted; however, the data were inadequate to assess whether these pharmacological effects would also occur at lower levels of dietary exposure, which could lead to adverse effects in some individuals (e.g., those with hypotension or diabetes). The Committee allocated a temporary ADI, pending submission of further data on the pharmacological effects of steviol glycosides in humans. A temporary ADI of 0 – 2 mg per kg bw was established for steviol glycosides, expressed as steviol, based on a NOEL for stevioside of 970 mg per kg bw per day (or 383 mg per kg bw per day, expressed as steviol) in the 2-year study in rats and a safety factor of 200.
- 68th Meeting (WHO, 2007)– Further data were submitted showing the purity of at 95% and all steviol glycosides hydrolyze to steviol upon ingestion. JECFA determined that it was unnecessary to maintain a limit for the sum of stevioside and rebaudioside content that could include product that was at least 95% stevioside or at least 95% rebaudioside A. The Chemical and Technical Assessment report, written after the 2007 meeting, explained the Committee’s thinking, which resulted in flexibility in the identity specifications (FAO, 2007a; FAO, 2007b)
- 69th Meeting (WHO, 2008) – Based on additional clinical studies, JECFA finalized the evaluation of steviol glycosides and raised the ADI to 0 – 4 mg per kg bw per day and removed the “temporary” designation. A summary of the Committee’s key conclusions was published in the final toxicology monograph addendum (WHO, 2009).

B. Summary of FSANZ Review of Steviol Glycosides

- In 2008, FSANZ reviewed the safety of steviol glycosides and concluded that they are well-tolerated and unlikely to have adverse effects on blood pressure, blood glucose, or other parameters in normal, hypotensive, or diabetic subjects at doses up to 11 mg per kg bw per day. FSANZ agreed with JECFA in setting an ADI of 4 mg steviol equivalents per kg bw per day (FSANZ, 2008).
- On May 13, 2011, FSANZ approved an increase in the maximum permitted level (MPL) of steviol glycosides (expressed as steviol equivalents) in ice cream, water based beverages, brewed soft drinks, formulated beverages and flavored soy beverages up to 200 mg per kg and in plain soy beverages up to 100 mg per kg (FSANZ, 2011).
- On January 16, 2016, FSANZ approved the addition of rebaudioside M as a steviol glycoside intense sweetener (FSANZ, 2016a).

- On February 20, 2017, FSANZ broadened the definition and hence specification for steviol glycosides preparations to include any mixture of individual steviol glycosides extracted from the stevia leaf.

C. Summary of EFSA Review of Steviol Glycosides

- On March 10, 2010, EFSA adopted a scientific opinion on the safety of steviol glycosides (mixtures that comprise not less than 95% of stevioside and/or rebaudioside A) as a food additive based upon JECFA's 2008 findings and in response to the European Commission's request to reevaluate the safety of steviol glycosides as a sweetener (EFSA, 2010).
 - EFSA agreed that the results of toxicology studies on either stevioside or rebaudioside A are applicable for the safety assessment of steviol glycosides.
 - EFSA established an ADI for steviol glycosides, expressed as steviol equivalents, of 4 mg per kg bw per day primarily based on the application of a 100-fold uncertainty factor to the NOAEL in the 2-year carcinogenicity study in the rat when administering 2.5% stevioside in the diet (Toyoda et al., 1997).
- On January 11, 2011, EFSA revised the exposure assessment of steviol glycosides from its use as a food additive, for children and adults, based on the revised proposed uses presented.
 - EFSA reduced usage levels in 16 foods by a factor of 1.5 to 3, with no changes for 12 food groups.
 - The mean estimated exposure to steviol glycosides (equivalents) in European children (aged 1-14 years) ranged from 0.4 to 6.4 mg per kg bw per day and from 1.7 to 16.3 mg per kg bw per day at the 95th percentile.
 - A correction was considered to be necessary for the consumption of non-alcoholic flavored drinks (soft drinks) by children, and the corrected exposure estimate at the 95th percentile for children ranged from 1.0 to 12.7 mg per kg bw per day.
 - For adults, the mean and 97.5th percentile intakes were estimated to range from 1.9 to 2.3 and 5.6 to 6.8 mg per kg bw per day, respectively.
 - These revised exposure estimates to steviol glycosides remain above the established ADI of 4 mg per kg bw (steviol equivalent).

D. Other Published Reviews

- Stevia and steviol glycosides have been extensively investigated for their biological, toxicological, and clinical effects (Carakostas et al., 2008; Geuns, 2003; Huxtable, 2002).
- Four additional reviews have appeared on the toxicology and biological activity of stevia extracts and steviol glycosides (Yadav and Guleria, 2012; Brown and Rother, 2012; Brahmachari et al., 2011; Chatsudthipong and Muanprasat, 2009). The studies are not always closely comparable because:
 - These reviews do not clearly differentiate between studies on crude stevia extract and purified steviol glycosides.
 - Studies on biological activity used routes of administration other than oral

- Some studies may have used doses that are much higher than anticipated human use levels.
- Roberts and Munro (2009) criticized the Chatsudthipong and Muanprasat (2009) review with points that are applicable – in general – to all the reviews:
 - Lack of purity of the material,
 - Route of exposure in relation to metabolism and safety assessment - *in vitro* and intravenous, intraperitoneal, or subcutaneous dosing studies are not relevant to the safety of steviol glycosides consumed orally.
 - Paucity of discussion of worldwide regulatory authorities affirming the safety of purified forms of stevioside and rebaudioside A as a food ingredient.
- In 2015, Urban et al. reviewed the potential allergenicity of steviol glycosides. The authors noted that: “hypersensitivity reactions to stevia in any form are rare” and concluded that current data do not support claims that steviol glycosides are allergenic. In addition, the authors stated that there is “little substantiated scientific evidence” to warrant consumer warning statements to consumers about allergy to highly purified stevia extracts.

Appendix 15 GRAS Associates Expert Panel Report

The Generally Recognized as Safe (GRAS) Status of the Proposed Uses of High Purity Glucosylated Steviol Glycosides Preparations

April 18, 2019

Foreword

An independent panel of experts (“Expert Panel”) was convened by GRAS Associates, LLC on behalf of their clients, Qufu Shengren Pharmaceutical Co., Ltd. (hereinafter “Qufu”), Sunwin Stevia International (hereinafter “Sunwin”), and NuNaturals, Inc. (hereinafter “NuNaturals”) to evaluate the safety and Generally Recognized as Safe (GRAS) status of Qufu, Sunwin, and NuNaturals’s proposed uses of TasteRight Enzyme Treated Stevia and Refined Enzyme Treated Stevia preparations in conventional foods. The members of this Expert Panel[†] are qualified to serve in this capacity by qualification of scientific training and experience in the safety of food and food ingredients.

Discussion

A significant amount of safety information related to the consumption of steviol glycosides is generally available, and has been discussed in Part 6, as well as Appendices 11-14, of Qufu, Sunwin, and NuNaturals’s dossier. First, there is a history of safe consumption of steviol glycosides when used as an ingredient in food products in the U.S., Canada, South America, Europe, Asia, and Australia and New Zealand. Second, a number of experimental studies have investigated the safety of steviol glycosides. The composite evidence from historical safe consumption and experimental studies collectively demonstrate the safety of the subject enzyme modified high purity steviol glycosides preparations for human food consumption.

The majority of the studies reviewed on steviol glycosides and steviol have been discussed in detail in previous GRAS submissions, including GRN 555, GRN 548, and GRN 536.

With regard to the safety documentation, the key pharmacokinetic data establish that steviol glycosides are not absorbed through the GI tract, *per se*; they are converted to steviol by bacteria normally present in the large intestine, and the steviol is absorbed but rapidly metabolized to steviol glucuronide and excreted. It has been well-established experimentally from various published studies that the steviol glycoside molecules are not absorbed from the GI tract (Gardana et al., 2003; Koyama et al., 2003b). The action of bacteria in the large intestine is directly

[†] Dr. Emmel, Chair of the Expert Panel, is a chemist with substantial food safety experience in addressing steviol glycosides and other food ingredients. Dr. Kapp is a toxicologist with over 35 years of experience. He is a Fellow of the Academy of Toxicological Sciences, a Fellow of the Royal Society of Biology, and a European Registered Toxicologist. Dr. Lewis is a biologist with more than 10 years of experience preparing GRAS dossiers. All three panelists have extensive technical backgrounds in the evaluation of food ingredient safety and in participating in deliberations of GRAS Expert Panels.

supported by the published study that showed that steviol glycosides can be converted to steviol in the large intestine by normal anaerobic GI flora as demonstrated by an *in vitro* study in fecal homogenates (Koyama et al., 2003a; Renwick and Tarka, 2008). The ADI for steviol glycosides has been set largely based on a published chronic study in rats (Toyoda et al., 1997) and several published clinical studies that show there are no pharmacological effects in humans at doses several fold higher than the ADI (Barriocanal et al., 2006; Barriocanal et al., 2008; Wheeler et al., 2008). Recently, Roberts et al. (2016) noted in a persuasive argument using a chemical-specific adjustment factor (CSAF) that the ADI could be higher. The toxicity of the metabolite steviol has been well reviewed in the published literature (Geuns, 2003; WHO, 2006; Urban et al., 2013). In addition, FDA has issued “no questions” letters to 56 GRN submissions for steviol glycosides preparations, including 7 notifications regarding enzyme modified steviol glycosides.

The Expert Panel notes that Qufu, Sunwin, and NuNaturals’s manufacturing process for their high purity glucosylated glycoside preparations is similar to the processes described for other GRAS enzyme modified steviol glycosides materials, as described in GRN 337, GRN 375, GRN 448, GRN 452, GRN 607, GRN 656, and GRN 662. The updated scientific literature review of steviol glycosides covering the time frame since GRN 662 was submitted through the present revealed no findings raising new safety concerns that would alter the previous GRAS determinations for similar enzyme modified steviol glycosides preparations.

The GRAS Associates Expert Panel convened on behalf of Qufu, Sunwin, and NuNaturals has reviewed the proposed uses for TasteRight Enzyme Treated Stevia and Refined Enzyme Treated Stevia. The highest 90th percentile consumption by any population subgroup of TasteRight was calculated to be approximately 9.90 mg per kg bw per day (for TasteRight Enzyme Treated Stevia), which is equivalent to 3.66 mg per kg bw per day steviol equivalents (calculated by a weighted sum estimate) for any population group, on a worst-case scenario basis. This estimated intake value is well below the JECFA ADI of 4 mg per kg bw per day expressed as steviol equivalents. Therefore, TasteRight Enzyme Treated Stevia and Refined Enzyme Treated Stevia are expected to be safe within established allowable limits.

A compelling case can be made that scientific consensus exists regarding the safety of steviol glycosides when of sufficiently high purity. The central role of conversion to steviol and subsequent elimination with these naturally occurring steviol glycosides extends to the manner in which the various steviol glycosides molecules are metabolized and eliminated from the body. While the scientific conclusions are not unanimous regarding the safe human food uses of steviol glycosides, the Panel believes that a wide consensus does exist in the scientific community to support a GRAS conclusion as evidenced by several publications (Carakostas, 2012; Geuns, 2007; Urban et al., 2013; Waddell, 2011; Williams, 2007; Brusick, 2008) that refute safety concerns expressed by a minority of scientists. In addition, Roberts et al. (2016) suggest that the ADI for steviol glycosides could be as high as 6-16 mg per kg bw per day, which is higher than has been previously accepted by the scientific community, providing the potential for an even more robust safety profile.

In summary, sufficient qualitative and quantitative scientific evidence in the composite is available to support the safety-in-use of Qufu, Sunwin, and NuNaturals’s TasteRight Enzyme Treated Stevia and Refined Enzyme Treated Stevia high purity steviol glycosides ($\geq 95\%$ total steviol glycosides and glucosylated steviosides) preparation given the following conditions:

- Qufu, Sunwin, and NuNaturals’s TasteRight Enzyme Treated Stevia and Refined Enzyme Treated Stevia high purity glucosylated steviol glycosides preparations continue to meet the designated specifications;
- the minimum sweetness intensities for TasteRight Enzyme Treated Stevia and Refined Enzyme Treated Stevia remain unchanged; and
- the high purity glucosylated steviol glycosides are produced in accordance with Current Good Manufacturing Practices (CGMPs).

Conclusion

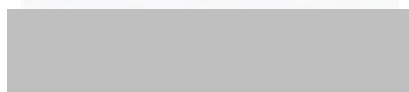
The Expert Panel critically reviewed the data provided by Qufu, Sunwin, and NuNaturals for their TasteRight Enzyme Treated Stevia and Refined Enzyme Treated Stevia high purity glucosylated steviol glycosides preparations, as well as publicly available published information obtained from peer-reviewed journals and other safety assessments prepared by other Expert Panels and well-respected international regulatory bodies.

The ingestion of Qufu, Sunwin, and NuNaturals’s TasteRight Enzyme Treated Stevia and Refined Enzyme Treated Stevia from the intended uses results in intakes that are safe within the limits of established historical use and published safety studies and the widely accepted ADI of 4 mg per kg bw per day steviol equivalents.

The Expert Panel unanimously concluded that the proposed uses of Qufu, Sunwin, and NuNaturals’s TasteRight Enzyme Treated Stevia and Refined Enzyme Treated Stevia preparations, manufactured as described in Part 2.B. of their dossier, and declared within the subject notification meet the FDA definition of safety in that there is “reasonable certainty of no harm under the intended conditions of use” as described herein, and Qufu, Sunwin, and NuNaturals’s TasteRight Enzyme Treated Stevia and Refined Enzyme Treated Stevia ($\geq 95\%$ total steviol glycosides and glucosylated steviosides) preparations are generally recognized as safe (GRAS).



Robert W. Kapp, Jr., Ph.D.
Fellow ATS, FRSB, & ERT(UK).



Kara Lewis, Ph.D.



Katrina Emmel, Ph.D.
Panel Chair

END

From: [Katrina Emmel](#)
To: [Zhang, Janet](#)
Cc: [Amy Mozingo](#); [William J. Rowe](#)
Subject: Re: GRN 000858
Date: Tuesday, August 6, 2019 11:44:09 PM
Attachments: [FDA Response Ltr GRN 858 August 6 2019.pdf](#)

Hello Dr. Zhang,

Attached you will find a response letter addressing the questions provided in your email on July 29, 2019 regarding GRN 885. Please let me know if you have any further questions.

Thank you,

Katrina

Katrina Emmel, Ph.D.
Senior Scientist/Project Manager/Associate
GRAS Associates, LLC.

emmel@gras-associates.com

On Aug 1, 2019, at 2:17 PM, Zhang, Janet <Janet.Zhang@fda.hhs.gov> wrote:

Thanks, Katrina.

Best regards,

Janet

Jianrong (Janet) Zhang, Ph.D.

FDA/OFVM/CFSAN/OFAS/DST

College Park, MD 20740

Phone: 240-402-1327

janet.zhang@fda.hhs.gov

<[image001.png](#)>

<[image002.jpg](#)> <[image003.jpg](#)> <[image004.jpg](#)> <[image005.jpg](#)> <[image006.jpg](#)>

From: Katrina Emmel <emmel@gras-associates.com>

Sent: Thursday, August 1, 2019 1:44 PM

To: Zhang, Janet <Janet.Zhang@fda.hhs.gov>

Cc: Amy Mozingo <amozingo@gras-associates.com>; William J. Rowe
<wrowe@nutrasource.ca>

Subject: GRN 000858

Dear Dr. Zhang,

I am confirming receipt of FDA's questions regarding GRN 858, which were email to William Rowe on July 29, 2019 (copied below). Will is currently out of the office, so I can serve as the main point of contact for you on this going forward.

We are currently coordinating with Qufu, Sunwin, and NuNaturals to provide you a response on or before August 12, 2019. Please feel free to contact me directly should you have any further questions or concerns regarding this GRN.

Thank you,

Katrina

Katrina Emmel, Ph.D.
Senior Scientist/Project Manager/Associate
GRAS Associates, LLC.

emmel@gras-associates.com

From: "Zhang, Janet" <Janet.Zhang@fda.hhs.gov>

Date: July 29, 2019 at 11:39:19 PM GMT+2

To: "William J. Rowe" <wrowe@nutrasource.ca>

Subject: GRN 000858

Dear Mr. Rowe:

We note that the specification for the content of total steviol glycosides listed in Table 4 of your notice is =95%, however, the specified limit for dextrin is up to 20%. Please provide the specified minimum for total steviol glycosides on a whole dry weight basis for the ingredient. Further, please clarify that the analytical results for total steviol glycosides in Table 4 and the certificates of analysis in Appendix 3 of your notice reflect concentrations for the whole ingredient on a dry weight basis. We note that in Appendix 2, Table 2.2 of your notice, the specification for glucosyl steviol glycosides is =75% and up to 20% for maltodextrin.

Thanks,

Janet

Jianrong (Janet) Zhang, Ph.D.

FDA/OFVM/CFSAN/OFAS/DST

College Park, MD 20740

Phone: 240-402-1327

janet.zhang@fda.hhs.gov



GRAS Associates, LLC
27499 Riverview Center Blvd.
Bonita Springs, FL 34134
T: 239.444.1724 | F: 239.444.1723
www.gras-associates.com

August 6, 2019

Food and Drug Administration
Center for Food Safety & Applied Nutrition
Office of Food Additive Safety (HFS-255)
5001 Campus Drive
College Park, MD 20740-3835

Attention: Dr. Janet Zhang

Re: GRN 858 –High Purity Steviol Glycosides –Response to Questions Posed in an Email
Dated 7/29/19

Dear Dr. Zhang:

Per your request, GRAS Associates, LLC, acting as the agent for Qufu Shengren Pharmaceutical Co., Ltd. (“Qufu”, People’s Republic of China), Sunwin Stevia International (“Sunwin”, USA), and NuNaturals, Inc. (“NuNaturals”, USA), is providing a response to complete FDA’s request for additional information as denoted in your email dated July 29, 2019, as follows:

We note that the specification for the content of total steviol glycosides listed in Table 4 of your notice is $\geq 95\%$, however, the specified limit for dextrin is up to 20%. Please provide the specified minimum for total steviol glycosides on a whole dry weight basis for the ingredient. Further, please clarify that the analytical results for total steviol glycosides in Table 4 and the certificates of analysis in Appendix 3 of your notice reflect concentrations for the whole ingredient on a dry weight basis. We note that in Appendix 2, Table 2.2 of your notice, the specification for glucosyl steviol glycosides is $\geq 75\%$ and up to 20% for maltodextrin.

Qufu, Sunwin, and NuNaturals are providing revised Certificates of Analyses to clarify that the TasteRight Enzyme Treated Stevia preparation described in GRN 858 is a mixture of ~80% total steviol glycosides and glucosylated steviol glycosides and ~20% maltodextrin. Furthermore, the purity of the total steviol glycosides in the TasteRight Enzyme Treated Stevia finished product is determined by HPLC after removing the maltodextrin from the mixture, for which the minimum specification has been set at $\geq 95\%$ total steviol glycosides and glucosylated steviol glycosides. Results are reported on a dry weight basis. Table 4, below, has been updated accordingly to reflect these clarifications, and the revised COAs are provided in Appendix A of this letter.



GRAS Associates, LLC
 27499 Riverview Center Blvd.
 Bonita Springs, FL 34134
 T: 239.444.1724 | F: 239.444.1723
www.gras-associates.com

Revised Table 1. Specifications for Qufu, Sunwin, and NuNaturals’s TasteRight Enzyme Treated Stevia Preparation

PHYSICAL & CHEMICAL PARAMETERS	JECFA ^a SPECIFICATIONS STEVIOL GLYCOSIDES	FCC ^b SPECIFICATIONS REBAUDIOSIDE A	QUFU, SUNWIN, & NUNATURALS’S MINIMUM SPECIFICATIONS FOR TASTERIGHT ENZYME TREATED STEVIA	RESULTS OF TASTERIGHT ENZYME TREATED STEVIA PREPARATIONS				
				BATCH NUMBER 20170302	BATCH NUMBER 20170305	BATCH NUMBER 20170401	BATCH NUMBER 20170502	BATCH NUMBER 20170703
Appearance Form	Powder	Crystal, granule or powder	Powder	Pass	Pass	Pass	Pass	Pass
Appearance Color	White to light Yellow	White to off-white	White to off-white	Pass	Pass	Pass	Pass	Pass
Sweetness Intensity ^c	--	--	100-150	110	110	110	110	110
Solubility	Freely soluble in water: ethanol (50:50)	Freely soluble in water: ethanol (50:50)	Freely soluble in water: ethanol (50:50)	Meets specification	Meets specification	Meets specification	Meets specification	Meets specification
Purity (HPLC Area) %	≥95 Steviol Glycosides	≥ 95	≥ 95 Total Steviol Glycosides ^d	99.07	99.74	99.17	99.26	99.74
			≥ 75 Glucosylated steviol glycosides ^d	81.20	80.78	82.10	81.37	82.78
Total Steviol Glycosides %	NA	NA	≥ 80	81.96	80.99	82.79	81.98	83.00
Dextrin (%)	NA	NA	≤ 20	18.04	19.01	17.21	18.02	17.00
Residual Ethanol	NMT 5,000 mg/kg	NMT 0.5%	≤ 5,000 ppm	249 ppm	272 ppm	270 ppm	266 ppm	199 ppm
Residual Methanol	NMT 200 mg/kg	NMT 0.02%	≤ 200 ppm	32 ppm	26 ppm	53 ppm	50 ppm	30 ppm
Loss on Drying	NMT 6.0%	NMT 6.0%	≤ 6%	2.57%	2.65%	2.66%	2.68%	2.70%
pH, 1% Solution	4.5-7.0	4.5-7.0	4.5-7.0	5.86	5.83	5.80	5.76	5.70
Total Ash	NMT 1%	NMT 1%	≤ 1 %	0.71%	0.71%	0.71%	0.71%	0.71%
Arsenic	NMT 1 mg/kg	NMT 1 mg/kg	≤ 1 ppm	≤ 1 ppm	≤ 1 ppm	≤ 1 ppm	≤ 1 ppm	≤ 1 ppm
Lead	NMT 1 mg/kg	NMT 1 mg/kg	≤ 1 ppm (as total heavy metals)	≤ 1 ppm	≤ 1 ppm	≤ 1 ppm	≤ 1 ppm	≤ 1 ppm



Total Plate Count (cfu/g, max)	NMT 1,000	NA	< 1,000	< 1,000	< 1,000	< 1,000	< 1,000	< 1,000
Yeast & Mold (cfu/g, max)	NMT 200	NA	< 100	< 100	< 100	< 100	< 100	< 100
<i>E. coli</i> (mpn/g)	Negative in 1 g	NA	Negative	Negative	Negative	Negative	Negative	Negative
<i>Salmonella spp.</i>	Negative in 25 g	NA	Negative in 25 g	Negative	Negative	Negative	Negative	Negative

^a Prepared at 84th JECFA (2017)

^b Rebaudioside A monograph. Food Chemicals Codex (7th Ed.) (FCC, 2010)

^c Compared with a 2% sucrose solution

^d HPLC analysis performed after removal of maltodextrin from the sample.

NA = not applicable; NMT = not more than; mg = milligram; g = gram; kg = kilogram; cfu = colony forming unit; ppm = part per million; mpn = most probable number; HPLC = high-performance liquid chromatography



GRAS Associates, LLC
27499 Riverview Center Blvd.
Bonita Springs, FL 34134
T: 239.444.1724 | F: 239.444.1723
www.gras-associates.com

If additional information or clarification is needed as you and your colleagues proceed with the review, please feel free to contact me via email.

We look forward to your feedback.

Sincerely,



Katrina V. Emmel, Ph.D.
Senior Scientist/Associate
GRAS Associates, LLC
27499 Riverview Center Blvd., Suite 212
Bonita Springs, FL 34134
emmel@gras-associates.com

Appendix 1 Revised Certificates of Analysis for Multiple Batches of TasteRight Enzyme Treated Stevia

Appendix 1.1 TasteRight Enzyme Treated Stevia Batch 20170305

Appendix 1.2 TasteRight Enzyme Treated Stevia Batch 20170401

Appendix 1.3 TasteRight Enzyme Treated Stevia Batch 20170502

Appendix 1.4 TasteRight Enzyme Treated Stevia Batch 20170703

Appendix 1.5 TasteRight Enzyme Treated Stevia Batch 20170302

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Appendix 1.1 TasteRight Enzyme Treated Stevia Batch 20170305



圣仁制药
 Shengren Pharmaceuticals

Analysis Report of Glucosyl Stevia

文件编号 File No. REG-ZL-0005-00

产品 PRODUCT

名称 Name	酶改质甜菊糖 Enzyme Treated Stevia
规格 Specification	E.T.S.
批号 Batch NO.	20170305
生产日期 Date of production	Mar. 05, 2017
批量 Batch quantity	1000kg

描述 DESCRIPTION

外观 Appearance	White powder
有效期限 Expiration date	2years
包装 Packing	Carton
贮存 Storage conditions	Keep in cool, dry and ventilating place

纯度 PURITY

参数 Parameter	标准 Standard	结果 Results
总含量% Total content of Stev Glycosides%	≥80	80.99
甜菊糖纯度% Purity of Steviol Glycosides%	≥95	99.74
葡萄糖基甜菊糖含量% Glucosyl steviol glycosides%	≥75	80.78
还原糖%Dextrin%	≤20	19.01

化学检测 CHEMICAL TESTS

参数 Parameter	标准 Standard	结果 Results
PH	4.5-7.0	5.83
甲醇 ppmMethanolppm	≤200	26
乙醇 ppmEthanolppm	≤5000	272
砷 Arsenic (以 As 计) ppm	≤1	≤1
重金属 Pb Total heavy metals ppm	≤1	≤1
目数 Mesh Thru #40	通过 95% NLT95%	96%
甜度 Sweetness	≥100	Meet the specification
堆积密度 Density-Bulk	0.20-0.40g/ml	0.310
密度 Density-Tapped	0.30-0.6g/ml	0.340
灼烧残渣% Ash	≤4.5	0.71
干燥失重% Loss on drying	≤6	2.65
需氧菌总数 TABC	<1000cfu/g	<1000cfu/g
霉菌及酵母菌数 Yeast/Mold	<100 cfu/g	<100 cfu/g
大肠杆菌 E.Coli	Negative	Negative
沙门氏菌 Salmonella	Negative	Negative

结论: 合格

Conclusion: Qualified for sales and human consumption.

检验: *王*
 Analyst

审核: *费*
 Auditing

日期: 2017 年 3 月 10 日

日期: 2017 年 3 月 17 日

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Appendix 1.2 TasteRight Enzyme Treated Stevia Batch 20170401



圣仁制药
 Shengren Pharmaceuticals

Analysis Report of Glucosyl Stevia

文件编号 File No. REG-ZL-0005-00

产品 PRODUCT

名称 Name	酶改质甜菊糖 Enzyme Treated Stevia
规格 Specification	E.T.S.
批号 Batch NO.	20170401
生产日期 Date of production	Apr. 15, 2017
批量 Batch quantity	1000kg

描述 DESCRIPTION

外观 Appearance	White Powder
有效期限 Expiration date	2Years
包装 Packing	Carton
贮存 Storage conditions	Keep in cool, dry and ventilating place

纯度 PURITY

参数 Parameter	标准 Standard	结果 Results
总含量 Total content of Steviol Glycosides	≥ 80	82.79
甜菊糖纯度% Purity of Steviol Glycosides	≥ 95	99.17
葡萄糖基甜菊糖含量% Glucosyl steviol glycosides%	≥ 75	82.10
还原糖 Dextrin %	≤ 20%	17.21

化学检测 CHEMICAL TESTS

参数 Parameter	标准 Standard	结果 Results
PH	4.5-7.0	5.80
甲醇 ppmMethanolppm	≤ 200	53
乙醇 ppmEthanolppm	≤ 5000	270
砷 Arsenic (以 As 计) ppm	≤ 1	≤ 1
重金属 Pb Total heavy metals ppm	≤ 1	≤ 1
目数 Mesh Thru #40	通过 95% NLT95%	96%
甜度 Sweetness	≥ 100	Meet the specification
堆积密度 Density-Bulk	0.20-0.40g/ml	0.315
密度 Density-Tapped	0.30-0.60g/ml	0.336
灼烧残渣% Ash	≤ 1	0.71
干燥失重% Loss on drying	≤ 6	2.66
需氧菌总数 TABC	< 1000cfu/g	< 1000cfu/g
霉菌及酵母菌数 Yeast/Mold	< 100 cfu/g	< 100 cfu/g
大肠杆菌 E.Coli	Negative	Negative
沙门氏菌 Salmonella	Negative	Negative

结论: 合格

Conclusion: Qualified for sales and human consumption.

检验:

Analyst

日期: 2017 年 4 月 20 日

审核:

Auditing

日期: 2017 年 4 月 27 日

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Appendix 1.3 TasteRight Enzyme Treated Stevia Batch 20170502



圣仁制药
 Shengren Pharmaceuticals

Analysis Report of Glucosyl Stevia

文件编号 File No. REG-ZL-0005-00

产品 PRODUCT

名称 Name	酶改质甜菊糖 Enzyme Treated Stevia
规格 Specification	E.T.S
批号 Batch NO.	20170502
生产日期 Date of production	May 02, 2017
批量 Batch quantity	1000kg

描述 DESCRIPTION

外观 Appearance	White Powder
有效期限 Expiration date	2years
包装 Packing	Carton
贮存 Storage conditions	Keep in cool, dry and ventilating place

纯度 PURITY

参数 Parameter	标准 Standard	结果 Results
总含量% Total content of Steviol Glycosides	≥ 80	81.98
甜菊糖纯度% Purity of Steviol Glycosides	≥ 95	99.26
葡萄糖基甜菊糖含量% Glucosyl steviol glycosides%	≥ 75	81.37
还原糖 Dextrin%	≤ 20%	18.02

化学检测 CHEMICAL TESTS

参数 Parameter	标准 Standard	结果 Results
PH	4.5-7.0	5.76
甲醇 ppm Methanol ppm	≤ 200	50
乙醇 ppm Ethanol ppm	≤ 5000	266
砷 Arsenic (以 As 计) ppm	≤ 1	≤ 1
重金属 Pb Total heavy metals ppm	≤ 1	≤ 1
目数 Mesh Thru #40	通过 95% NLT 95%	96%
甜度 Sweetness	≥ 100	Meet the specification
堆积密度 Density-Bulk	0.20-0.40g/ml	0.314
密度 Density-Tapped	0.30-0.60g/ml	0.336
灼烧残渣% Ash	≤ 6	0.71
干燥失重% Loss on drying	≤ 6	2.68
需氧菌总数 TABC	< 1000cfu/g	1000cfu/g
霉菌及酵母菌数 Yeast/Mold	< 100 cfu/g	< 100 cfu/g
大肠杆菌 E.Coli	Negative	Negative
沙门氏菌 Salmonella	Negative	Negative

结论: 合格

Conclusion: Qualified for sales and human consumption.

检验: 王华
 Analyst


日期: 2017 年 5 月 7 日

审核: 贾月
 Auditing

日期: 2017 年 5 月 14 日

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Appendix 1.4 TasteRight Enzyme Treated Stevia Batch 20170703



圣仁制药

Shengren Pharmaceuticals

Analysis Report of Glucosyl Stevia

文件编号 File No. REG-ZL-0005-00

产品 PRODUCT

名称 Name	酶改质甜菊糖 Enzyme Treated Stevia
规格 Specification	E.T.S.
批号 Batch NO.	20170703
生产日期 Date of production	Jul. 03, 2017
批量 Batch quantity	1000kg

描述 DESCRIPTION

外观 Appearance	White Powder
有效期限 Expiration date	2years
包装 Packing	Carton
贮存 Storage conditions	Keep in cool, dry and ventilating place



纯度 PURITY

参数 Parameter	标准 Standard	结果 Results
总含量% Total content of Steviol Glycosides	≥80	83.00
甜菊糖纯度% Purity of Steviol Glycosides	≥95	99.74
葡萄糖基甜菊糖含量% Glucosyl steviol glycosides%	≥75	82.78
还原糖% Dextrin%	≤20%	17.00

化学检测 CHEMICAL TESTS

参数 Parameter	标准 Standard	结果 Results
PH	4.5-7.0	5.70
甲醇 ppm Methanol ppm	≤200	30
乙醇 ppm Ethanol ppm	≤5000	199
砷 Arsenic (以 As 计) ppm	≤1	≤1
重金属 Pb Total heavy metals ppm	≤1	≤1
目数 Mesh Thru #40	通过 95% NLT 95%	96%
甜度 Sweetness	≥100	Meet the specification
堆积密度 Density-Bulk	0.20-0.40g/ml	0.315
密度 Density-Tapped	0.30-0.60g/ml	0.338
灼烧残渣% Ash	≤1	0.71
干燥失重% Loss on drying	≤6	2.70
需氧菌总数 TABC	<1000 cfu/g	<100 cfu/g
霉菌及酵母菌数 Yeast/Mold	<100 cfu/g	<100 cfu/g
大肠杆菌 E.Coli	Negative	Negative
沙门氏菌 Salmonella	Negative	Negative

结论: 合格
Conclusion: Qualified for sales and human consumption.

检验:  Analyst
 审核:  Auditing

日期: 2017 年 7 月 8 日
 日期: 2017 年 7 月 15 日

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Appendix 1.5 TasteRight Enzyme Treated Stevia Batch 20170302



Analysis Report of Glucosyl Stevia

文件编号 File No. REG-ZL-0005-00

产品 PRODUCT

名称 Name	酶改质甜菊糖 Enzyme Treated Stevia
规格 Specification	E.T.S.
批号 Batch NO.	20170302
生产日期 Date of production	Mar.02,2017
批量 Batch quantity	1000kg

描述 DESCRIPTION

外观 Appearance	White powder
有效期限 Expiration date	2 Years
包装 Packing	Carton
贮存 Storage conditions	Keep in cool, dry and ventilating place

纯度 PURITY

参数 Parameter	标准 Standard	结果 Results
总含量% Total content of Steviol Glycosides%	≥ 80	81.96
甜菊糖纯度% Purity of Steviol Glycosides%	≥ 95	99.07
葡萄糖基甜菊糖含量% Glucosyl steviol glycosides%	≥ 75	81.20
还原糖% Dextrin%	≤ 20	18.04

化学检测 CHEMICAL TESTS

参数 Parameter	标准 Standard	结果 Results
PH	4.5-7.0	5.86
甲醇 ppm Methanol ppm	≤ 200	32
乙醇 ppm Ethanol ppm	≤ 5000	249
砷 Arsenic (以 As 计) ppm	≤ 1	≤ 1
重金属 Pb Total heavy metals ppm	≤ 1	≤ 1
目数 Mesh Thru #40	通过 95% NLT 95%	96%
甜度 Sweetness	≥ 100	Meet the specification
堆积密度 Density-Bulk	0.20-0.4g/ml	0.316
密度 Density-Tapped	0.30-0.6g/ml	0.339
灼烧残渣% Ash	≤ 1	0.71
干燥失重% Loss on drying	≤ 6.0%	5.37
需氧菌总数 TABC	< 1000 cfu/g	< 1000 cfu/g
霉菌及酵母菌数 Yeast/Mold	< 100 cfu/g	< 100 cfu/g
大肠杆菌 E.Coli	Negative	Negative
沙门氏菌 Salmonella	Negative	Negative

结论: 合格

Conclusion: Qualified for sales and human consumption.

检验: 孙金峰
Analyst

审核: 贾二有
Auditing

日期: 2017 年 3 月 7 日

日期: 2017 年 3 月 14 日