The Coca-Cola Company	ANALYTICAL REPORT
Title : Screening for Leachates from Resins in REBM	Date: 09-05-14

REPORT PREPARED BY			
Name	**Department**	**Signature**	**Date**
[redacted]	Global Research	[redacted]	9-10-14
[redacted]	Global Research		9/10/14
	Global Research		9-10-14

REPORT APPROVED BY			
Name	**Department**	**Signature**	**Date**
[redacted]	Global Research	[redacted]	9-10-14

Key Words: REBM, resin, leachate

1.0 Abstract:

Samples of REBM50 and REBM95 were analyzed for styrene, divinylbenzene (DVB), trimethylamine (TMA) and triethylamine (TEA) as potential leachates from the resins used to purify the steviol glycosides. For all samples, all of the analytes were below the detection limits, which are 0.1 mg/kg or less. Styrene and DVB were analyzed by P&T-GC-MS. In the case of TMA and TEA, LC-MS-MS was used for the analysis.

2.0 Background:

Rebaudioside M (REBM) is a novel, high-intensity, natural sweetener from the Stevia *rebaudiana* Bertoni perennial shrub originally found in South America. As part of the processing of the REBM-containing extracts, they are purified using anion-exchange resins made of styrene and divinylbenzene. We were requested to analyze samples of REBM50 and REBM95 for styrene and divinylbenzene, as well as trimethylamine and triethylamine, all of which are potential leachates from the resins.

3.0 Materials and Methods:

3.1 Starting Material:.

Both the RebM95 (Lot#s PT310714, Lot# PT050814 and PT130814) and RebM50 (Lot#s PT300714, PT240714 and PT170513) samples are from PureCircle, Ltd. (Negeri Sembilan, Malaysia).

3.2 LC-MS-MS:

3.2.1 Standard Preparation:

Trimethylamine hydrochloride (TMA HCl) and triethylamine hydrochloride (TEA HCl), both from Sigma, were used as standards for this study. Standards were prepared in the manner of weight-to-weight (w/w). Separate stock solutions were prepared by weighing 0.02 g of each solid into separate 40 mL glass amber vials, and then adding 0.15% formic acid to bring the total weight to 20 g, which yielded a solution of 1000 mg/kg concentration (before correcting for moisture, purity, and formula weigh conversion).

In the second step, TMA HCl and TEA HCl solutions were mixed and diluted 1000-fold by combining 0.03 g of each stock solution in a new glass amber vial, and then filling with 0.15% formic acid to a total weight of 30 g. This yielded a combined TMA HCl and TEA HCl solution with 1 mg/kg of each.

The 1 ppm solution was further diluted 1000X and 10,000X with 1000 ppm REBM 95 solution to prepare 1 µg/kg and 0.1 µg/kg solution. The 1000 ppm

REBM 95 solution was prepared by dissolving 0.1 g control REBM 95 (known that free of the testing leachates) in 100 mL of 0.15% formic acid.

The actual concentrations were calculated based on the actual weights, moisture content, % purity, and formula weigh conversion to account for HCl.

Calibration curves were generated by variable injection, as detailed in the following table.

Calibration Level	Solution Conc.	Injection Volume	Final Corrected Conc., ppb	
			TMA	TEA
Level 1	0.1 ppb	10 µL	0.028	0.040
Level 2	0.1 ppb	20 µL	0.056	0.080
Level 3	1 ppb	2 µL	0.057	0.082
Level 4	1 ppb	10 µL	0.284	0.411
Level 5	1 ppb	20 µL	0.569	0.821

For method validation, the solutions were prepared in a similar manner. Two concentration levels were used for method validation, 0.05 ppb and 0.5 ppb.

3.2.2 Sample Preparation:

The REBM 50 and REBM 95 samples were prepared in triplicate by dissolving 0.02 g of samples in 20 g of 0.15% formic acid.

To ensure the accuracy of testing, each sample is also spiked at two levels, 0.1 ppb and 0.8 ppb concentrations.

3.2.3 LC/MS/MS on 5500 QTRAP:

3.2.3.1 LC Conditions:
Isocratic run: 20% of 1% formic acid in water (mobile A) and 80% methanol (B).
Flow rate: 0.3 mL/min
Column: Waters Atlantis HILIC 2.1 mm X 150 mm, 5 micron
Column Temperature: 35 °C
Injection volumn: 20 µL
Run time: 5 min

3.2.3.2 MS Conditions:
Ionization mode: Positive

Curtain gas: 20
Collision gas: Medium
IonSpray voltage: 4500
Temperature: 300
Ion source gas 1: 20
Ion source gas 2: 60
Switch valve: 1-1.8 min to waste, 1.8-5 min to MS
MRM transitions:

Precursor	Product	Time (ms)	ID	Declustering	Entrance	Collision	Collision cell exit
60.1	44	150	TMA	90	4	24	7
69.1	49	150	TMA-D9	105	10	27	10
102.1	57.9	150	TEA	90	5	26	8
117.1	65	150	TEA-D15	185	5	41	10

3.3 P&T-GC-MS:

3.3.1 Sample and Standard Preparation:

Weigh 0.01 g of each sample into a 40 mL EPA vial. Then add one small drop of antifoam emulsion and 10.0 ml of DI water. Cap the vial immediately for analysis. To the vial, 1 µL of 10000 µg/L styrene-d8 is added by the P&T auto-sampler as the internal standard.

To prepare the divinylbenzene and styrene stock standards, weigh them into separate volumetric flasks and then fill them to the mark with P&T grade methanol. Mix styrene and divinylbenzene stock standards in methanol to make 10000 µg/L, 1000 µg/L and 100 µg/L calibration working standard solutions. The calibration standard in water is made by following the worksheet below.

Styrene / DVB Working Standard Used	Styrene / DVB Working Standard Volume	Volume of Water or Matched Solution in 40 mL EPA Vial	Styrene/DVB Conc. In Calibration Standard
µg/L	µL	mL	µg/L
100	10	10	0.10
100	50	10	0.50
1000	5	10	0.50
1000	20	10	2.00
1000	50	10	5.00
10000	10	10	10.0
10000	25	10	25.0

3.3.2 Instrument Settings:

The Agilent 6890GC/5973MSD with EST Encon/8100 purge & trap system is used to analyze the standard and samples. A K trap is used for P&T trap sampling and the GC column is a Rtx-VMS 20 m X 0.18 mm X 1.0 µm. The MSD is run in the SIM mode for styrene-d8 (m/z 112 and 84), styrene (m/z 104 and 78), m-divinylbenzene and p-divinylbenzene (m/z 130, 129, 128 and 115). The Agilent ChemStation software is used for data processing.

4.0 Results & Discussion:

LC-MS-MS:

In all samples, trimethylamine (TMA) is less than 0.05 mg/kg and triethylamine (TEA) is less than 0.04 mg/kg, which are the limits-of-detection for each.

For spikes, each compound was spiked at 2 levels for each sample, 0.1 and 0.8 µg/kg in the 1000 ppm solution. Spike recoveries are good for both levels, ranging from 86% to 121% (most are between 90% and 110%). For method validation, reproducibility is good for TMA at 0.28 µg/kg and TEA at 0.08 and 0.41 ug/kg. Reproducibility was not as good for TMA at 0.05 µg/kg, mainly because it is close to its LOD.

P&T-GC-MS:

All 1000 mg/L water solutions of the samples had less than 0.1 µg/L styrene and divinylbenzene (DVB). Therefore, the levels of styrene and (DVB) in the samples were both below their detection limits (0.1 mg/kg).

For spikes, 0.5 µg/L of styrene and DVB were spiked in one of the test sample solutions and tested along with the samples. One of the control samples was spiked with 0.2 µg/L of styrene and DVB and tested 10 times. The recoveries ranged from 80% to 115%. Reproducibility for 0.2 µg/L spike was 2.4% (Styrene), 6.7% (m-DVB) and 7.2% (p-DVB).

5.0 Conclusion:

Samples of REBM50 and REBM95 have been analyzed for leachates from resins used to purify them. The targeted analyses were for trimethylamine (TMA) and triethylamine (TEA) by LC-MS-MS and styrene and divyinlybenzene (DVB) by purge-and-trap GC-MS. For all samples, all four analytes were below the detection limit for each, which is 0.05 mg/kg TMA, 0.04 mg/kg TEA, 0.1 mg/kg styrene and 0.1 mg/kg DVB. Both methods showed good accuracy for the target analytes.