

# HPLC Determination of Glycoside Sweeteners: Steviol Glycosides and Mogroside V

Ian Acworth,<sup>1</sup> Deanna Hurum,<sup>2</sup> Brian De Borba,<sup>2</sup> Jeffrey Rohrer,<sup>2</sup> and Deepali Mohindra,<sup>2</sup>  
<sup>1</sup>Thermo Fisher Scientific, Chelmsford, MA, USA; <sup>2</sup>Thermo Fisher Scientific, Sunnyvale, CA, USA

## Introduction

Terpene glycoside extracts of plants are gaining popularity as sweeteners and some, such as stevia extracts, have been successfully commercialized as off-the-shelf sugar substitutes. These compounds are noncaloric, but have been reported to have sweetness greater than 300 times the equivalent of sucrose solutions, making them attractive sugar replacements.<sup>1</sup> Both purified glycosides from plant extracts and whole fruit or leaf extracts are available on the market as sweeteners, herbal teas, or nutritional supplements.

Many glycosides are present in stevia (*Stevia rebaudiana* [Bertoni]) extracts, with rebaudioside A of primary commercial interest because it has been given Generally Recognized as Safe (GRAS) status by the U.S. FDA.<sup>2</sup> Mogroside V, another terpene glycoside sweetener, is extracted from the fruit of the lo-han-guo (*Siraitia grosvenorii*), extracts of which have also been given GRAS status.<sup>3</sup> Due to the structural similarity of such terpene glycosides—both from a single plant species and across species—chromatographic separation can be challenging.

FIGURE 1. Primary steviol glycosides of interest.

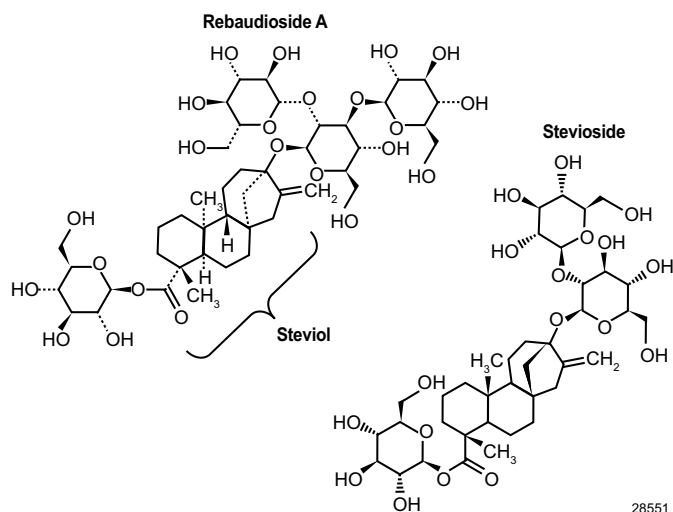
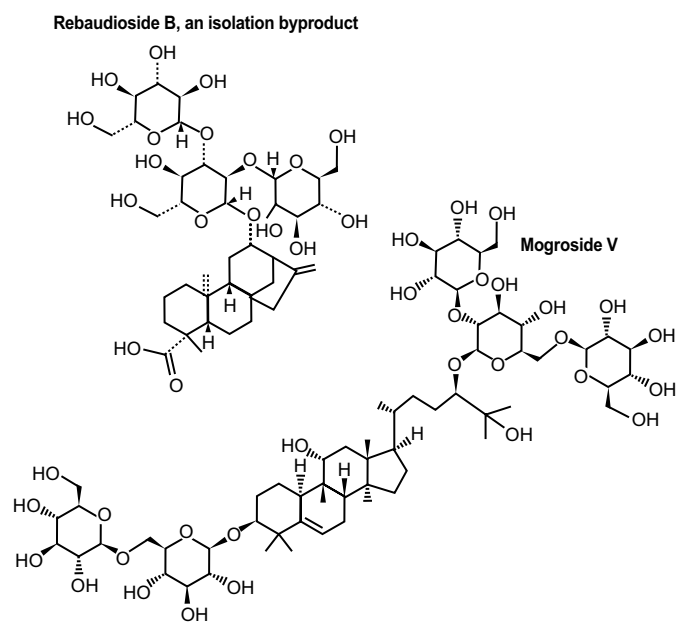
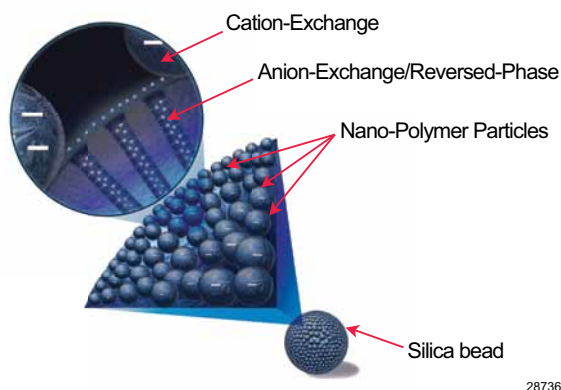


FIGURE 2. Additional terpene glycosides of interest.



This work demonstrates an isocratic method with a trimode column for the determination of terpene glycosides. The high volatility of the mobile phase makes the method ideal for an aerosolizing detection technique such as charged aerosol detection. By using the trimode column, the analytes of interest are rapidly separated with clear differentiation between steviol glycosides and mogroside V. This column incorporates spatially separated anion-exchange, cation-exchange, and reversed-phase functionalities with minimal stationary phase cross-functional interaction, allowing excellent control of analyte retention by adjusting the ionic strength, pH, and organic content of the mobile phase.

**FIGURE 3. Thermo Scientific Acclaim™ Trinity™ P1 column.**



Additionally, by using UV and charged aerosol detection the method provides greater flexibility in determining the relative proportion of glycosides. Two commercial stevia-based sweeteners and a lo-han-guo tea are analyzed here, with calibration ranges from 7–280 µg/mL. Advantages of using two detection techniques are illustrated using off-the-shelf samples with detection sensitivity by charged aerosol detection improved 3–4-fold for glycosides that do not strongly absorb in the UV range, such as rebaudioside B and mogroside V.

### Experimental Conditions

Thermo Scientific Dionex UltiMate™ 3000 RSLC system consisting of:

SRD-3600 Solvent Rack

HPG-3400RS Pump

WPS-3000TRS Autosampler

TCC-3000RS Column Compartment

DAD-3000RS Diode Array Detector

Thermo Scientific Dionex Corona™ *ultra*™ Charged Aerosol Detector

Column: Acclaim Trinity P1, 3 µm  
Analytical 2.1 × 100 mm  
Acclaim Trinity P1,  
3 µm Guard 2.1 × 10 mm

Mobile Phase: 81/19 acetonitrile/10 mM ammonium formate, pH= 3.00

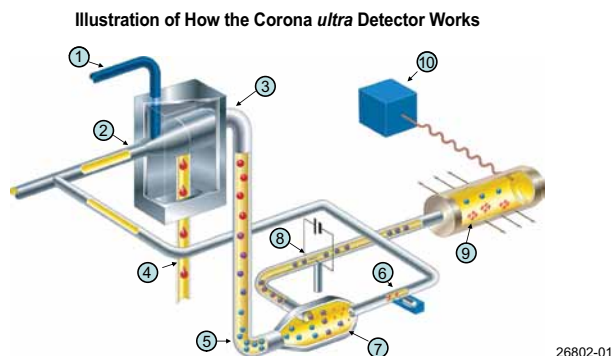
Flow Rate: 0.3 mL/min

Temperature: 20 °C

Inj. Volume: 5 µL

Detection: DAD UV-vis detector, 210 nm  
Dionex Corona *ultra* detector, filter = none  
Nebulizer temperature, 10 °C

**FIGURE 4. The liquid eluent from the HPLC column enters the Corona *ultra* detector (1) where it undergoes pneumatic nebulization (2). Small droplets enter the drying tube (3) and form particles, while large drops exit the drain (4) to waste. Dried particles enter the mixing chamber (5). Another gas stream passes over a charged Corona *ultra* needle (6). Charged gas then mixes with the dried particles, forming charged particles (7). High mobility species are removed by an ion trap (8) while the remaining charged particles pass to a collector where the charge is measured with a very sensitive electrometer (9). Signal is transferred to software (10).**



## Sample Preparation

### Stevia Sweeteners

Sweetener samples (0.100 g) were extracted with 10 mL of mobile phase. For those sweeteners with components that do not dissolve, the suspension was vortexed a minimum of 2 min total to ensure adequate mixing and extraction. A separate packet of sweetener was used for sample preparations each day. Sweeteners that exceeded the calibration curve were diluted by a factor of 4.

### Lo-Han-Guo Beverages

The lo-han-guo beverage is supplied as a pair of flavored sugar cubes (15.2 g). These were dissolved in 100 mL of deionized water. The sample was further diluted by a factor of 20 in acetonitrile. Samples which showed precipitates were filtered.

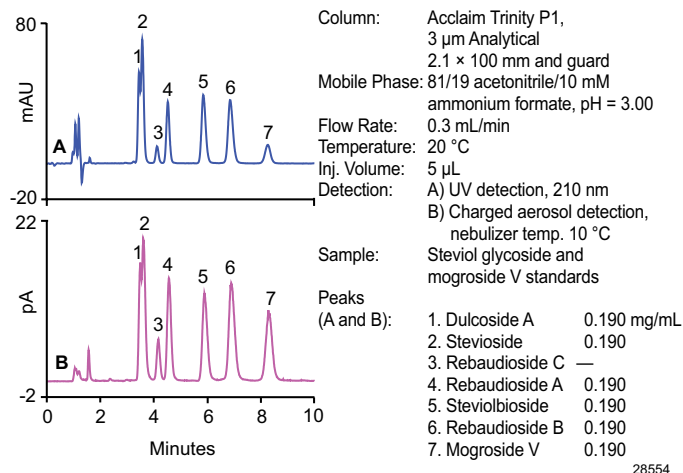
## Results and Discussion

The chromatogram in Figure 5 shows the separation of steviol glycoside and mogroside V standards. The separation of dulcoside A from stevioside can be controlled by increasing the amount of acetonitrile in the mobile phase. In this case, 81% acetonitrile was used to allow better separation of other components found in sweetener Brand A.

When using these mixed standards for calibration curves, the response is well correlated with the concentration. In this example, a quadratic calibration model was chosen for the charged aerosol detector data, although in a narrower calibration range (0.007–0.070 mg/mL) a linear calibration model works well ( $r^2 = 0.998$  for rebaudioside A).

The limits of quantification (LOQ) are lower by charged aerosol detection for all analytes, with greater sensitivity improvements for mogroside V, rebaudioside B, and rebaudioside A compared to the other glycosides.

FIGURE 5. Separation of standards.



28554

Table 1. Calibration (0.007–0.28 mg/mL for Each Glycoside), LOQ and Limits of Detection (LOD) Based on Injections of Standards

Analyte	Detector	LOQ ( $\mu$ g/mL)	LOD ( $\mu$ g/mL)	$r^2$	Calibration Model
Dulcoside A	UV: 210 nm	4.6	1.4	0.9997	linear
Dulcoside A	charged aerosol	2.3	0.7	0.9983	quadratic
Stevioside	UV: 210 nm	4.5	1.2	0.9996	linear
Stevioside	charged aerosol	1.4	0.4	0.9969	quadratic
Rebaudioside A	UV: 210 nm	7.0	2.3	0.9995	linear
Rebaudioside A	charged aerosol	2.3	0.7	0.9984	quadratic
Steviolbioside	UV: 210 nm	4.6	1.4	0.9996	linear
Steviolbioside	charged aerosol	2.3	0.7	0.9990	quadratic
Rebaudioside B	UV: 210 nm	7.0	2.3	0.9995	linear
Rebaudioside B	charged aerosol	2.3	0.7	0.9987	quadratic
Mogroside V	UV: 210 nm	22.0	7.0	0.9995	linear
Mogroside V	charged aerosol	4.6	1.4	0.9991	quadratic

## Precision

Precision of the method was evaluated by standard injection replicates (n = 7).

Table 2. Precision of Standard Injections (n = 7)					
Analyte	Detector	RT (min)	RT RSD	Peak Area (mAU*min) or (pA*min)	Peak Area RSD
Stevioside	UV: 210 nm	3.51	0.11	5.28	0.47
Stevioside	charged aerosol	3.54	0.24	2.15	0.70
Rebaudioside A	UV: 210 nm	4.42	0.14	2.90	0.58
Rebaudioside A	charged aerosol	4.46	0.18	1.62	0.53
Rebaudioside B	UV: 210 nm	6.30	0.09	4.62	0.87
Rebaudioside B	charged aerosol	6.33	0.21	2.30	0.75
Mogroside V	UV: 210 nm	8.03	0.16	0.926	1.67
Mogroside V	charged aerosol	8.07	0.07	1.72	0.75

Precision values calculated for seven injections of a 70 µg/mL standard of each of the glycosides.

Retention time precision and reproducibility have been reported as weaknesses of the current Joint FAO/WHO Expert Committee on Food Additives (JECFA) method for steviol glycoside determination. With the proposed method, consistent mobile phase preparation is critical to consistent retention times. Gravimetric mobile phase preparation is necessary. Retention time precision—measured as RSD—across multiple preparations of mobile phase was < 1.8 for rebaudioside A and < 1.4 for stevioside. Peak area RSDs for triplicate sample injections were < 1.0, which was equivalent to or better than peak area RSDs for seven standard injections.

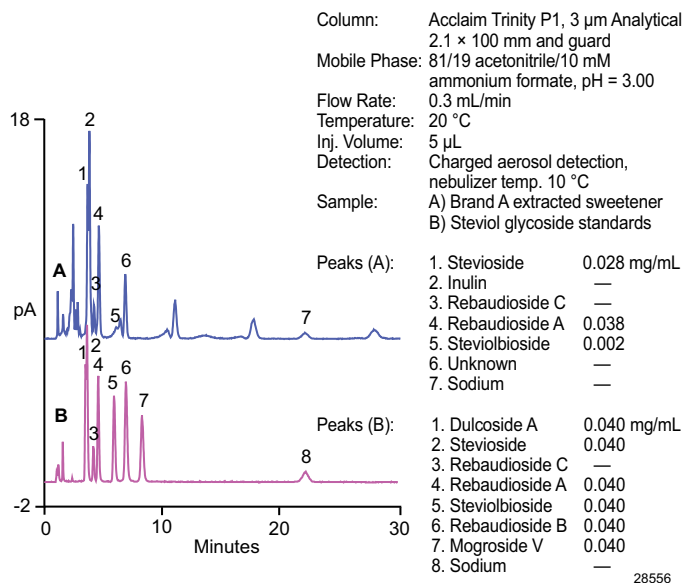
## Analysis of Stevia Sweeteners

Brand A, a sweetener composed of stevia leaf extract and inulin, was analyzed by the method described. Charged aerosol detection results were good, even for this highly complex sample. Due to the minimal processing of this product, a 30 min run time is recommended to allow elution of the many components (Figure 6).

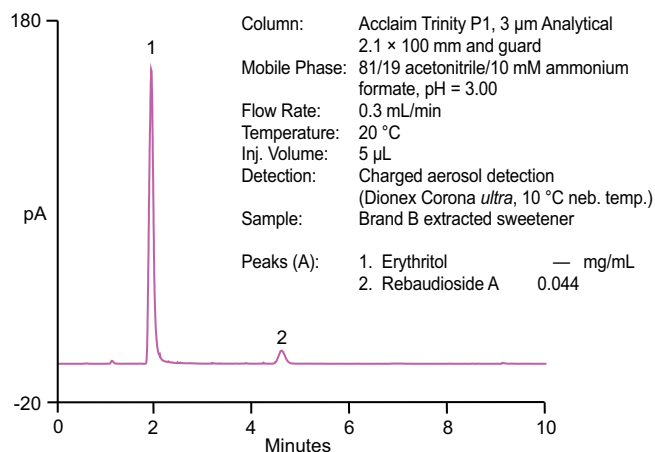
Brand B, a sweetener of rebaudioside A and erythritol, is a much simpler matrix. This sample can be analyzed in 10 min if the presence of sodium is controlled by using only polypropylene labware during sample preparation. The wide dynamic range of charged aerosol detection allows both the erythritol and the rebaudioside A to be detected under the same conditions (Figure 7).

The high sensitivity allows better determination of low-level impurity components, such as rebaudioside B (Figure 8).

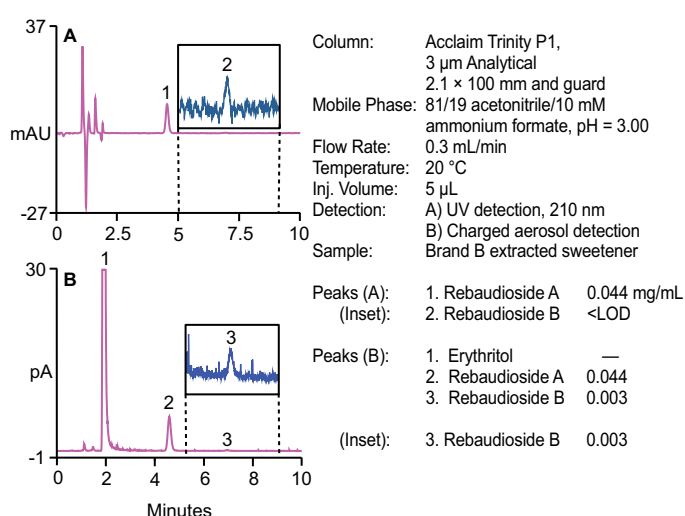
FIGURE 6. Analysis of brand A sweetener.



**FIGURE 7. Analysis of brand B sweetener.**



**FIGURE 8. Analysis of brand B sweetener.**



Precision of the assay was evaluated during three days of sample analysis with between day precision as RSD ranging from 2.3–13%.

**Table 3. Intra- and Between-Day Precision of Sweetener Analysis (n = 3)**

Day/ Sample	Analyte	Detector	Average mg Analyte/g Sweetener	Intraday Precision (RSD)	Between-Day Precision (RSD)
Day 1 Brand A	Rebaudioside A	UV	13	4.97	2.3
	Rebaudioside A	charged aerosol	15	1.51	4.5
	Stevioside	UV	12	5.12	1.6
	Stevioside	charged aerosol	10	6.25	10
Day 2 Brand A	Rebaudioside A	UV	14	4.64	
	Rebaudioside A	charged aerosol	15	4.56	
	Stevioside	UV	12	3.95	
	Stevioside	charged aerosol	11	1.88	
Day 3 Brand A	Rebaudioside A	UV	14	5.32	
	Rebaudioside A	charged aerosol	16	10.86	
	Stevioside	UV	12	3.13	
	Stevioside	charged aerosol	12	10.10	
Day 1 Brand B	Rebaudioside A	UV	3.9	4.4	12
		charged aerosol	4.2	5.1	13
Day 2 Brand B	Rebaudioside A	UV	3.6	6.9	
		charged aerosol	3.8	6.7	
Day 3 Brand B	Rebaudioside A	UV	4.8	7.1	
		charged aerosol	5.1	8.5	

## Accuracy

Rebaudioside A recoveries range from 85–104% by charged aerosol detection and 82–102% by UV detection over the three-day period. Stevioside recoveries range from 81–98% by charged aerosol detection and 84–105% by UV detection over the same period.

## Mogroside V in Lo-Han-Guo Beverages

Sucrose, a major component of the beverages tested, can potentially interfere with mogroside V determination. With the method shown here, sucrose elutes early and mogroside V is well retained. Mogroside V can be detected by both UV (210 nm) or charged aerosol detection with equivalent quantification in this sample.

An expansion of the previous chromatogram is shown in Figure 10. Charged aerosol detection is more sensitive to mogroside V than UV detection. In addition to the lo-han guo beverage, a mixed glycoside standard detected using charged aerosol is shown for retention time reference. Mogroside V is easily identified.

FIGURE 9. Lo-han-guo beverage.

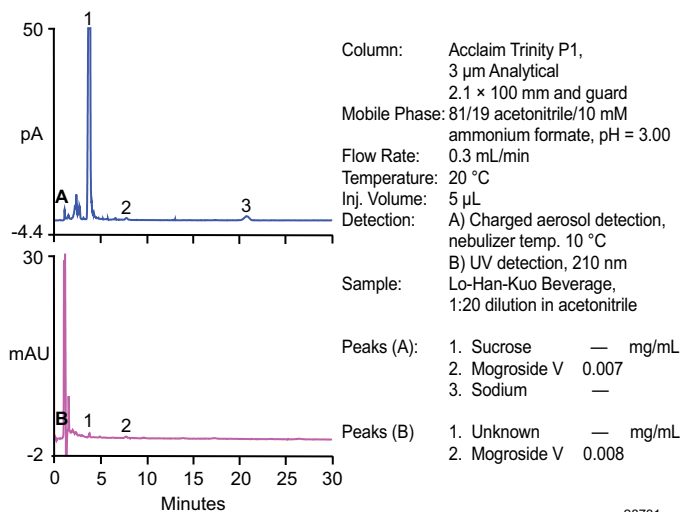
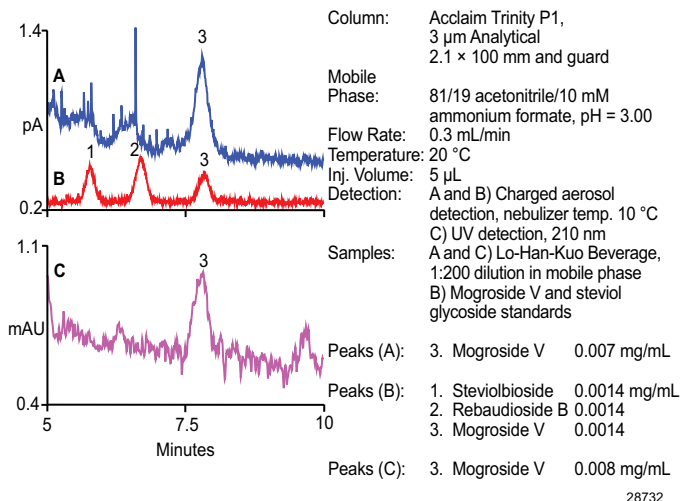


FIGURE 10. Lo-han-guo beverage—expansion around mogroside V peak.



The method proposed here is compared to existing methods for determination of rebaudioside A. As shown in the table below, current methods can be more time consuming and use a significant amount of mobile phase per sample analyzed.

**Table 4. Summary of Proposed Method, JECFA Monograph, and USP Monograph Conditions**

	<b>Proposed Method</b>	<b>JECFA Monograph<sup>4</sup></b>	<b>USP Monograph<sup>5</sup></b>
Separation Phase	Acclaim Trinity P1 3 µm Analytical 2.1 × 100 mm column	150–300 × 3.9–4.6 mm LC–NH <sub>2</sub> or equivalent	150 × 4.6 mm propyl amino silane bonded to silica gel, 5 µm
Mobile Phase	81:19 acetonitrile: 10 mM ammonium formate buffer, pH = 3.00	80:20 acetonitrile: water, phosphoric acid pH = 3.0	87:13 acetonitrile: acetate buffer
Detection	UV, 210 nm and charged aerosol	UV, 210 nm	UV, 210 nm
Stevioside RT	3.5 min	10 min	6.6 min
Rebaudioside A RT	4.4 min	21 min	14 min
Flow Rate (mL/min)	0.3	1–2 (flow rate set to meet rebaudioside A RT)	1.5
Analysis Time (min)	10–30	25	70
Mobile Phase per Sample (mL)	3.0–9.0	25–50	105

## Conclusions

Both UV and charged aerosol detection can be used to determine the studied terpene glycosides in sweeteners.

Charged aerosol detection is generally more sensitive by a factor of 2, although some differences between glycosides are observed, particularly for rebaudioside B and mogroside V.

This improved sensitivity makes charged aerosol detection useful in monitoring purification by-products in purified stevia sweeteners.

The method discussed is fast, allowing determination of the glycosides in 10 min. For whole leaf or fruit extracts, a 30 min run time is recommended, which still saves time and mobile phase compared to current methods.

## References

1. Prakash, I., DuBois, G.E.; Clos, J.F.; Wilkens, K.L.; Fosdick, L.E.; Development of Rebiana, a Natural, Non-Caloric Sweetener, *Food and Chemical Toxicology*, **2008**, *46*, S75–S82.
2. US Food and Drug Administration, Agency Response Letter to GRAS Notice No. GRN 000252, Dec 17, 2008.
3. US Food and Drug Administration, Agency Response Letter to GRAS Notice No. GRN 000301, Jan 15, 2010.
4. *Steviol Glycosides*. Prepared at the 69th JECFA (2008), published in FAO JECFA Monographs 5 (2008).
5. Rebaudioside A, Food Chemicals Codex, Seventh ed. Supplement 1, USPC, Inc. Washington DC, 2010.

©2011 Thermo Fisher Scientific, Inc. All other trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others.

**Dionex Products:** 1228 Titan Way, PO Box 3603, Sunnyvale, CA 94088-3603, (408) 737-0700  
**North America:** U.S./Canada (847) 295-7500  
**South America:** Brazil (55) 11 3731 5140  
**Europe:** Austria (43) 616 51 25, Benelux (31) 20 683 9768 (32) 3 353 4294  
Denmark (45) 36 36 90 90, France (33) 1 39 30 01 10, Germany (49) 61125 991 0  
Ireland (353) 644 0064, Italy (39) 02 51 62 1267, Sweden (46) 8 473 3380,  
Switzerland (41) 62 205 9966, United Kingdom (44) 1276 691722  
**Asia Pacific:** Australia (61) 2 9420 5233, China (852) 2428 3282, India (91) 22 2764 2735,  
Japan (81) 6885 1213, Korea (82) 2 2653 2580, Singapore (65) 6289 1190,  
Taiwan (886) 2 875 6655