APPLICATIONS

Increased Capabilities for the Analysis of Hormones in Drinking and Waste Water Using Solid Phase Extraction (SPE) and LC/MS/MS

Xianrong (Jenny) Wei, Sean Orlowicz, and Kristen Parnell
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA

Xianrong (Jenny) Wei

Senior Scientist
Jenny is a Senior Scientist in Phenomenex's PhenoLogix applications laboratory.

A fast and effective method is developed for expanded hormone analysis from drinking and waste water, which uses an optimized LC/MS/MS method with polarity switching within one injection, reducing analysis time from 55 minutes down to 13.5 minutes while maintaining excellent compound sensitivity, linearity and SPE recovery. The method uses a Phenomenex Strata® C18-E, 1 g / 20 mL SPE cartridge and Kinetex® 5 µm EVO C18 100 x 2.1 mm column with high pH mobile phase, without compromising column lifetime, while increasing a compound's sensitivity significantly. The lowest detection limits on a SCIEX API 4000™ QTRAP® could be reached at 0.05 ng/L for most analytes.

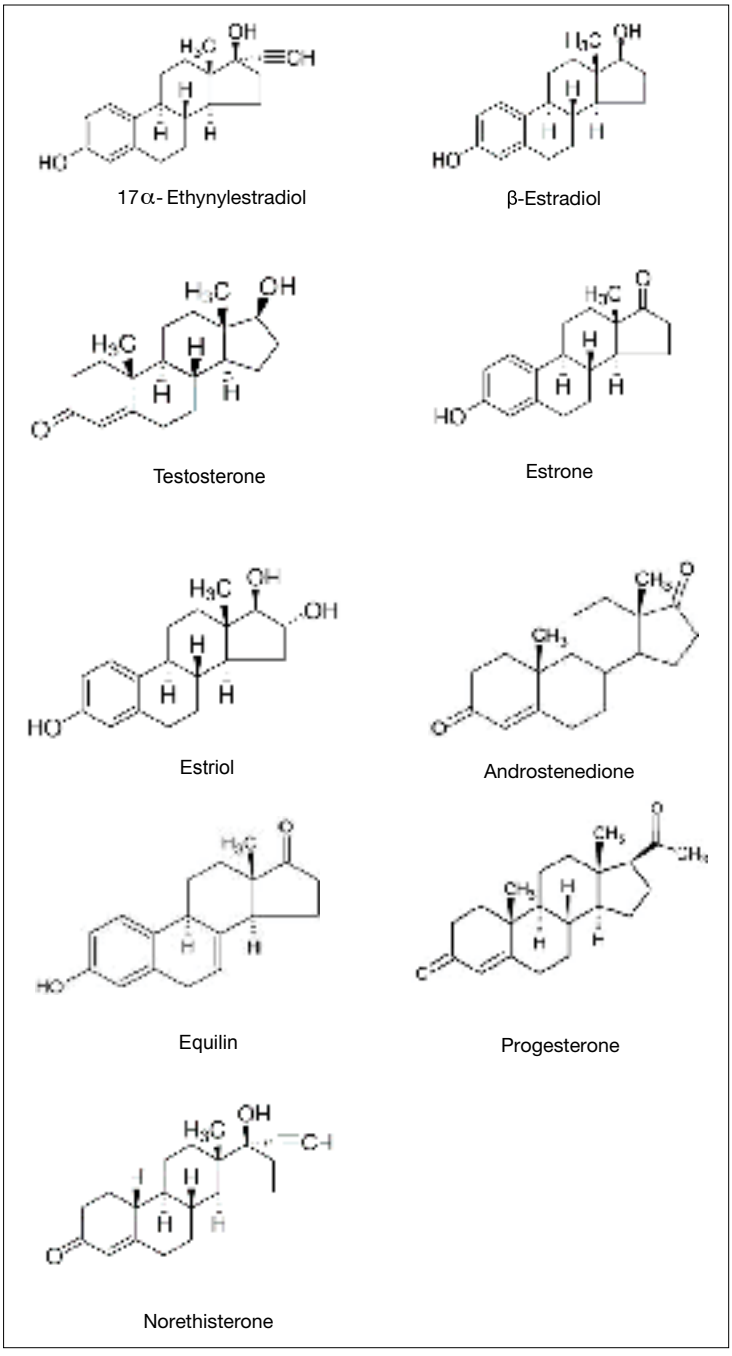
Introduction

The presence of various hormones in worldwide drinking water supplies has been of public concern for some time. As the scientific community tries to identify acceptable exposure limits, many new endogenous and synthetic hormones are being discovered. Compounds, such as ethynylestradiol, the active ingredient in a commonly prescribed birth control medication, are known to cause detrimental effects to both aquatic and human life. In addition, many other compounds are currently being investigated. Due to this public risk, the United States specifically developed EPA Method 539 to monitor this growing problem.

This study builds on the foundation of EPA 539 by providing an expanded list of target analytes to include compounds of current interest (e.g. progesterone) and a more modern extraction and separation method, all while shortening the run time to less than 14 minutes. We expand the sample preparation technique to employ a more versatile format for ease of use in a variety of water matrices. By utilizing a SPE tube format and exploring various particle sizes, differing water sources can be scaled and processed more easily. In addition, the final extract concentration levels can be easily monitored and controlled to be more appropriate for a variety of different detectors, depending on the required sensitivity.

This study also offers an optimized LC/MS/MS method that explores ionization polarities and utilizes a high pH mobile phase in order to maximize LC/MS/MS response. Because conventional silica-based HPLC media is not stable under alkaline conditions, a core-shell organo-silica column was used to perform these analyses.

Figure 1.
Structures of hormones tested in the study.

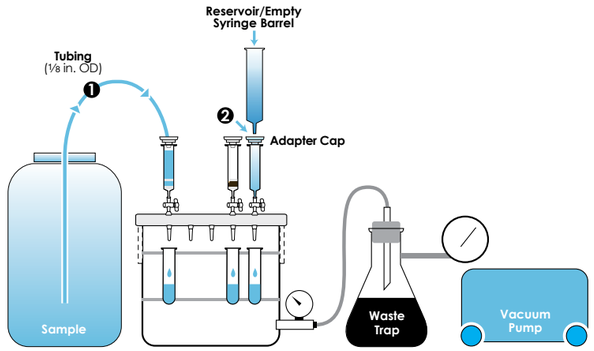


Experimental Conditions

Sample Preparation Parameters

Sample Pre-Treatment

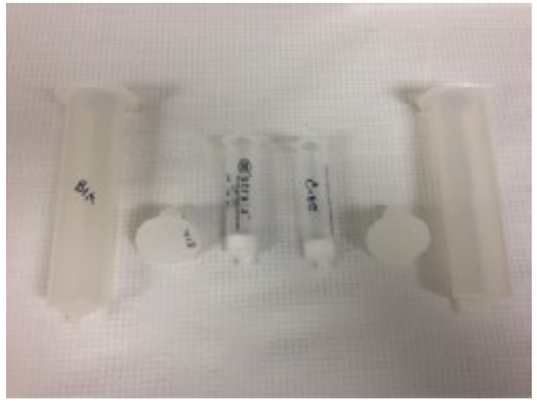
Per the EPA 539 method protocol, 1 L water samples are dechlorinated, preserved, collected, and stored. All standards are freshly prepared in 50 % methanol in water containing 20 ng/mL of working internal standards. Samples are processed using the below method and set up as demonstrated.



Optimized SPE Method

Cartridge: Strata® C18-E, 1 g / 20 mL
Part No.: 8B-S001-JEG
Condition: 10 mL methanol
Equilibrate: 10 mL water
Load: Pre-treated samples
Wash: 10 mL 15 % methanol in water
Dry: 5 – 10 minutes under 10" Hg vacuum
Elute: 2 x 6 mL methanol
Dry Down: Evaporate completely under a stream of nitrogen @ 50 °C
Reconstitute: Add 1.0 mL of 50 % methanol in water containing 20 ng/mL of working internal standards

SPE accessories and setup used for processing.



LC/MS/MS Parameters

Analytical Conditions

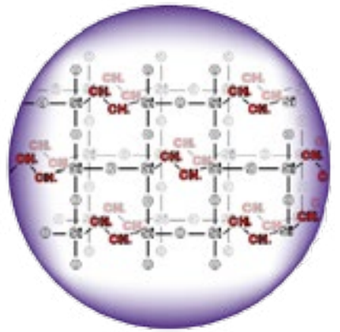
Column: Kinetex® 5 µm EVO C18
Dimensions: 100 x 2.1 mm
Part No.: 00D-4633-AN
Mobile Phase: A: 0.2 % NH_4OH in Water
B: 0.2 % NH_4OH in Methanol
Gradient:

Time (min)	% B
0	30
1	65
6	65
6.5	85
10.5	85
11	30
13.5	30

Flow Rate: 200 µL/min
Injection Volume: 30 µL
Instrument: Shimadzu® 30-AD and Agilent® 1260 LC
Detection: SCIEX API 4000™ QTRAP®
SCIEX API 4500™
Sample:
1. Estriol
2. Equilin
3. Estrone $^{13}C_3$
4. Estrone
5. 17β – Estradiol D_5
6. β – Estradiol
7. 17α - Ethynylestradiol
8. Norethisterone
9. Androstenedione
10. Testosterone D_3
11. Testosterone
12. Progesterone D_9
13. Progesterone

Note: The ionization source was ESI with rapid polarity switching in positive and negative ion mode. Shimadzu 30-AD LC series, interfaced with a SCIEX API 4000 QTRAP, was for lowest detection level test only; all other experiments were done on Agilent 1260 LC series with a SCIEX API 4500 mass spectrometer.

Figure 2.
Structure of the organo-silica Kinetex® EVO C18 particle.



MRM Transitions

ID (Neg Ions)	Q1	Q3	RT (min)
β-Estradiol 1	271.1	145	6.34
β-Estradiol 2	271.1	183.3	6.34
Estriol 1	287.1	145.2	4.21
Estriol 2	287.1	171	4.21
Equilin 1	267.1	143	5.80
Equilin 2	267.1	223.1	5.80
Estrone 1	269	145.1	6.14
Estrone 2	269	143.2	6.14
17α-Ethynylestradiol 1	295.3	145	6.46
17α-Ethynylestradiol 2	295.3	183.3	6.46
17 β-Estradiol D_5 1	276.3	147	6.29
17 β-Estradiol D_5 2	276.3	187.1	6.29
Estrone $^{13}C_3$ 1	272.2	148.1	6.13
Estrone $^{13}C_3$ 2	272.2	162.1	6.13

ID (Pos Ions)	Q1	Q3	RT (min)
Androstenedione 1	287.1	97.1	6.56
Androstenedione 2	287.1	109	6.56
Testosterone 1	289.1	97.2	7.20
Testosterone 2	289.1	109.1	7.20
Testosterone D_3 1	292.2	97	7.19
Testosterone D_3 2	292.2	109	7.19
Progesterone 1	315.4	109.1	9.67
Progesterone 2	315.4	97.2	9.67
Norethisterone 1	299.1	109	6.61
Norethisterone 2	299.1	231	6.61
Progesterone D_9 1	324.4	100	9.61
Progesterone D_9 2	324.4	113	9.61

Results and Discussion

Based on the foundation of EPA Method 539, a fast and reproducible SPE method for analyzing an expanded hormones list **(Figure 1)** in drinking and waste water using LC/MS/MS was developed. Both silica-based (Strata® C18-E) and polymer-based (Strata-X) SPE sorbents were evaluated as possible options for sample preparation for the mini-validation study using deionized (DI) water **(Table 1)**.

Table 1.
Silica-based and polymer-based sorbent comparison of extracted matrix recovery at 500 ng/L from DI water.

ID	Strata C18-E (55 µm, 1g/20 mL)		Strata-X (33 µm, 1g/20 mL)	
	Recovery %	CV %	Recovery %	CV %
17 β-Estradiol	99.7	2.93	97.4	6.64
Estriol	99.9	3.60	93.8	5.48
Equilin	91.1	2.75	42.4	5.95
Estrone	99.3	2.17	54.8	6.39
17α-Ethynylestradiol	103	3.67	99.1	5.58
Testosterone	99.9	0.10	100	0.06
Androstenedione	99.2	0.46	60.9	2.70
Progesterone	91.8	1.56	28.0	4.54
Norethisterone	101	3.78	97.9	4.03

In this study, the final method used Strata C18-E in tube format to bring benefits such as faster processing and increased sensitivity, consistency, and extraction efficiency; this silica-based sorbent provided near 100 % recoveries of (91.1 – 103 %) across all compounds **(Table 2)**. Additionally, the adaptable SPE setup allowed for sample processing volumes of 150 mL for easier extraction.

Tap water samples were then collected (n=3, collected on March 26, 2015 in Torrance, CA 90501, USA). Samples contained all the possible contaminants, so the analyte recoveries were various, but within ± 30 % of acceptance criteria, respectively **(Table 3)**.

Table 3.
Extracted matrix recoveries for tap water using Strata C18-E (55 µm, 1 g / 20 mL).

ID (Neg Ions)	2 ng/L (Tap Water)	
	Recovery %	CV %
17 β-Estradiol	88.1	6.53
Estriol	109	1.00
Equilin	72.8	8.46
Estrone	89.6	7.23
17α-Ethynylestradiol	91.6	3.15
ID (Pos Ions)	**2 ng/L (Tap Water)**	
	Recovery %	**CV %**
Testosterone	100	1.24
Androstenedione	108	5.09
Progesterone	113	5.07
Norethisterone	104	2.07

The analytical method used a high pH mobile phase which greatly improved the ionization of some of the target compounds, resulting in improved LC/MS/MS sensitivity. Because conventional silica-based HPLC media is unstable under alkaline conditions, the choice of analytical columns is very limited. In this study, we selected a Kinetex® 5 µm EVO C18 column (100 x 2.1 mm), a core-shell organo-silica particle which incorporates uniform stabilizing ethane cross-linking and provides resistance to high pH attack, all while maintaining mechanical strength **(Figure 2)**. These benefits allow an alkaline mobile phase to be used without compromising column lifetime and increase compound sensitivity significantly. The lowest detection limits on a SCIEX API 4000™ QTRAP® could be reached at 0.05 ng/L for most analytes **(Table 4)**.

To improve sample analysis for high-throughput in the lab, we have optimized LC/MS/MS procedures to effectively analyze all target compounds in one injection within 13.5 minutes **(Figures 3, 4** and **5)**, with mass spectrometer polarity switching. E1, E2, and E3 estrogens are also well-separated. The representatives of linearity of analytes across all positive and negative analytes are shown in **Figures 6** and **7**.

Table 2.
Mini-validation study results.

ID	Final Concentration (ng/L)	Accuracy %								
		Testosterone	Androstenedione	Progesterone	Norethisterone	17 β-estradiol	Estriol	Equilin	Estrone	17 α-Ethnylestradiol
STD 1	0.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
STD 2	0.5	83.1	100	95.0	94.9	97.8	95.8	91.6	94.8	92.4
STD 3	1.0	105	100	94.8	100	98.2	92.6	94.5	94.8	89.0
STD 4	2.0	103	104	99.0	96.5	98.5	96.9	91.9	99.9	90.9
STD 5	10	104	107	102	100	99.8	94.0	95.7	94.8	94.8
STD 6	40	98.9	99.5	103	102	99.8	102	103	103	102
STD 7	50	100	98.8	97.2	98.2	100	100	99.2	98.8	101
QC L	2	104	105	97.2	101	101	103	90.8	102	99.8
QC M	20	103	103	93.5	93	103	99.7	96.9	99.6	102

Table 4.
Method limits and linearity for each analyte.

ID (Neg Ions)	MRL (ng/L)	LOD (ng/L)	Linearity (R^2)
β-Estradiol	0.50	0.05	1.0000
Estriol	0.50	0.05	0.9997
Equilin	0.50	0.05	0.9997
Estrone	0.50	0.05	0.9996
17α-Ethynylestradiol	0.50	0.10	0.9998
ID (Pos Ions)	**MRL (ng/L)**	**LOD (ng/L)**	**Linearity (R^2)**
Androstenedione	0.50	0.05	0.9997
Testosterone	0.50	0.05	0.9998
Progesterone	0.50	0.05	0.9996
Norethisterone	0.50	0.10	0.9998

MRL - Method reporting limit **LOD** - Lowest of detection level **Linear Regression** at 1/X

Figure 3.
Representative total ion chromatogram.

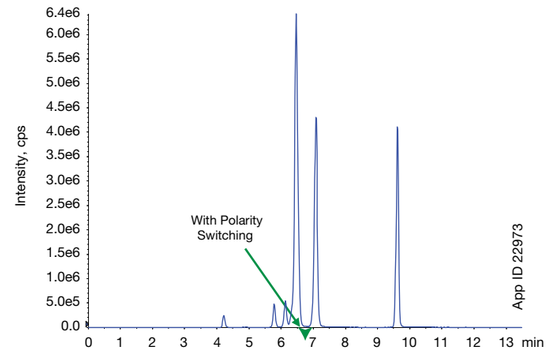


Figure 4.
Representative positive ion chromatogram – 2.0 ng/L.

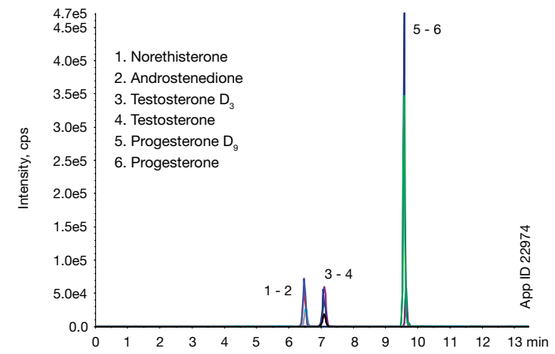


Figure 5.
Representative negative ion chromatogram – 2.0 ng/L.

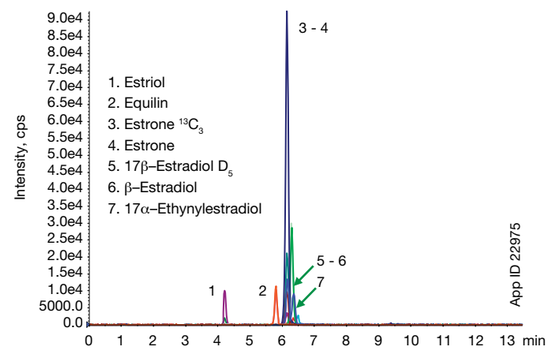


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Figure 6.
Representative Curve of Testosterone.

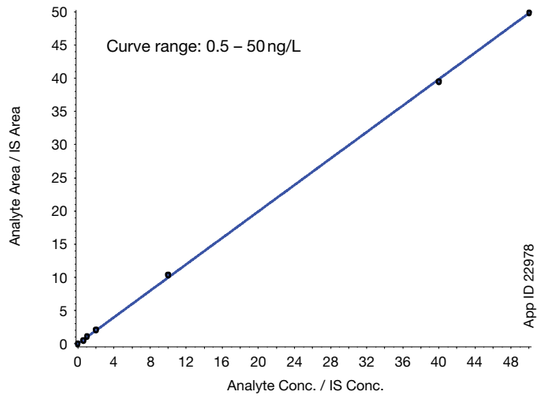
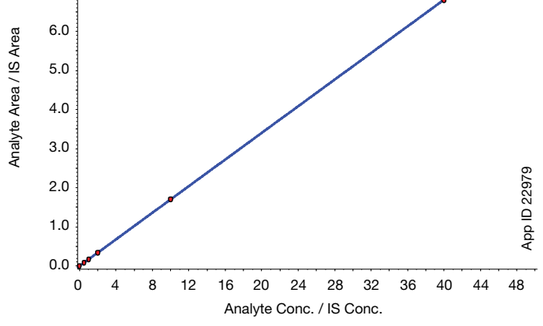


Figure 7.
Representative Curve of β-Estradiol.



Conclusions

EPA Method 539 is a liquid chromatography, electrospray ionization, and tandem mass spectrometry method for the determination of hormones in finished drinking water. This work presents an optimized method with an expanded list of hormones that includes compounds of current interest (e.g. progesterone and norethisterone) to successfully monitor a growing problem in our scientific community. An SPE extraction method using Phenomenex Strata® C18-E provides excellent recovery and reproducibility. LC/MS/MS analysis time was significantly reduced from 55 minutes to 13.5 minutes with a Kinetex 5 µm EVO C18 100 x 2.1 mm column, which delivers stability under high pH mobile phase, while maintaining excellent linearity and low detection levels. This method will significantly increase laboratory productivity, efficiency, and throughput.

Acknowledgements

We would like to provide special thanks to Julissa Fernandez for sample preparation and all PhenoLogix™ team members for their technical assistance.

TN-1184

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Ordering Information
Strata® C18-E

Format	Sorbent Mass	Part Number	Unit
Tube			
	1 g	8B-S001-JEG	20 mL (20/box)
	500 mg	8B-S001-HDG	12 mL (20/box)
	2 g	8B-S001-KDG	12 mL (20/box)
	5 g	8B-S001-LEG	20 mL (20/box)
	10 g	8B-S001-MFF	60 mL (16/box)
	20 g	8B-S001-VFF	60 mL (16/box)
	50 g	8B-S001-YSN	150 mL (8/box)
	70 g	8B-S001-ZSN	150 mL (8/box)

Ordering Information
Kinetex® EVO C18 Core-Shell LC Columns

5 µm Minibore Columns (mm)				SecurityGuard™ ULTRA Cartridges‡
30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
00A-4633-AN	00B-4633-AN	00D-4633-AN	00F-4633-AN	AJ0-9298 for 2.1 mm ID

5 µm MidBore™ Columns (mm)			SecurityGuard ULTRA Cartridges‡
50 x 3.0	100 x 3.0	150 x 3.0	3/pk
00B-4633-Y0	00D-4633-Y0	00F-4633-Y0	AJ0-9297 for 3.0 mm ID

5 µm Analytical Columns (mm)				SecurityGuard ULTRA Cartridges‡
50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	3/pk
00B-4633-E0	00D-4633-E0	00F-4633-E0	00G-4633-E0	AJ0-9296 for 4.6 mm ID

2.6 µm Minibore Columns (mm)				SecurityGuard™ ULTRA Cartridges‡
30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
00A-4725-AN	00B-4725-AN	00D-4725-AN	00F-4725-AN	AJ0-9298 for 2.1 mm ID

2.6 µm MidBore™ Columns (mm)			SecurityGuard ULTRA Cartridges‡
50 x 3.0	100 x 3.0	150 x 3.0	3/pk
00B-4725-Y0	00D-4725-Y0	00F-4725-Y0	AJ0-9297 for 3.0 mm ID

2.6 µm Analytical Columns (mm)			SecurityGuard ULTRA Cartridges‡
50 x 4.6	100 x 4.6	150 x 4.6	3/pk
00B-4725-E0	00D-4725-E0	00F-4725-E0	AJ0-9296 for 4.6 mm ID

1.7 µm Minibore Columns (mm)			SecurityGuard™ ULTRA Cartridges‡
50 x 2.1	100 x 2.1	150 x 2.1	3/pk
00B-4726-AN	00D-4726-AN	00F-4726-AN	AJ0-9298 for 2.1 mm ID

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

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Australia
t: +61 (0)2-9428-6444
f: +61 (0)2-9428-6445
auinfo@phenomenex.com

Austria
t: +43 (0)1-319-1301
f: +43 (0)1-319-1300
anfrage@phenomenex.com

Belgium
t: +32 (0)2 503 4015 (French)
t: +32 (0)2 511 8666 (Dutch)
f: +31 (0)30-2383749
beinfo@phenomenex.com

Canada
t: +1 (800) 543-3681
f: +1 (310) 328-7768
info@phenomenex.com

China
t: +86 (0)20 2282-6668
f: +86 (0)20 2809-8130
chinainfo@phenomenex.com

Denmark
t: +45 4824 8048
f: +45 4810 6265
nordicinfo@phenomenex.com

Finland
t: +358 (0)9 4789 0063
f: +45 4810 6265
nordicinfo@phenomenex.com

France
t: +33 (0)1 30 09 21 10
f: +33 (0)1 30 09 21 11
franceinfo@phenomenex.com

Germany
t: +49 (0)6021-58830-0
f: +49 (0)6021-58830-11
anfrage@phenomenex.com

India
t: +91 (0)40-3012 2400
f: +91 (0)40-3012 2411
indiainfo@phenomenex.com

Ireland
t: +353 (0)1 247 5405
f: +44 1625-501796
eireinfo@phenomenex.com

Italy
t: +39 051 6327511
f: +39 051 6327555
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

Mexico
t: 001-800-844-5226
f: 001-310-328-7768
tecnicomx@phenomenex.com

The Netherlands
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

New Zealand
t: +64 (0)9-4780951
f: +64 (0)9-4780952
nzinfo@phenomenex.com

Norway
t: +47 810 02 005
f: +45 4810 6265
nordicinfo@phenomenex.com

Puerto Rico
t: +1 (800) 541-HPLC
f: +1 (310) 328-7768
info@phenomenex.com

Spain
t: +34 91-413-8613
f: +34 91-413-2290
espinfo@phenomenex.com

Sweden
t: +46 (0)8 611 6950
f: +45 4810 6265
nordicinfo@phenomenex.com

United Kingdom
t: +44 (0)1625-501367
f: +44 (0)1625-501796
ukinfo@phenomenex.com

USA
t: +1 (310) 212-0555
f: +1 (310) 328-7768
info@phenomenex.com

All other countries
Corporate Office USA
t: +1 (310) 212-0555
f: +1 (310) 328-7768
info@phenomenex.com

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