

Automated Extraction and Determination of Human Hormones in Drinking Water Using Solid-Phase Extraction and HPLC with UV Detection

Carl Fisher and Linda Lopez
Thermo Fisher Scientific, Sunnyvale, California, USA

Keywords

Acclaim C18 column, EPA Method 539, estrogen, androgen, endocrine disruptor, Dionex AutoTrace 280

Goal

Demonstrate complete recovery of human hormones from drinking water using solvent extraction cartridges on a solid-phase extraction instrument

Introduction

The presence of hormones (from both natural and artificial sources) in drinking water is a human health concern.¹ Safe exposure limits have yet to be determined due to the need for further studies investigating the impact of long-term and synergistic exposure.² The European Parliament has identified several estrogen variants as priority substances that will be monitored to determine appropriate measures to address the risk posed by these compounds.³ Several hormones are routinely monitored as part of the U.S. Environmental Protection Agency (EPA) Unregulated Contaminant Monitoring program.⁴ These hormones include estriol, estrone, estradiol, ethynylestradiol, equilin, androstenedione, and testosterone (Figure 1).



They are structurally similar due to their common origin, the sterol cholesterol. Estriol, estrone, and estradiol are estrogens which control female traits, while testosterone is an androgen controlling the development of male characteristics. These hormones are naturally present in humans and are derived from androstenedione during steroidogenesis. Various forms of estrogen are prescribed as a hormonal contraceptive device for estrogen deficiency syndromes, and to counter the negative effects associated with the natural decline in estrogen levels, such as accelerated bone loss in postmenopausal women.⁵

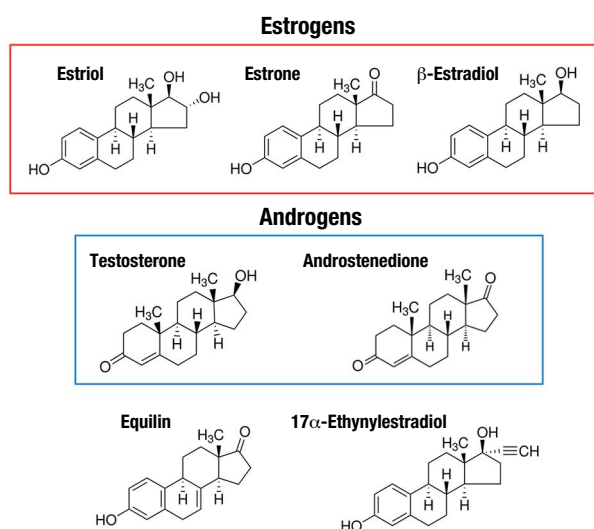


Figure 1. Sex hormones routinely monitored in drinking water. The naturally occurring human estrogens and androgens are highlighted.

Ethinylestradiol is an orally bioactive synthetic estrogen used as an oral contraceptive, while equilin is an equine-derived estrogen used for hormone replacement therapy. Testosterone is also prescribed for hormone replacement therapy in males whose natural levels have declined or are abnormally low.⁶ Androstenedione (also known as adione) was initially available as an over-the-counter supplement, but was banned by the International Olympic Committee in 1997 and placed in the category of androgenic-anabolic steroids. It was subsequently banned by the U.S. FDA in 2004 based on potential significant health risks similar to other anabolic steroids.⁷

Due to the widespread use of hormones in pharmaceuticals, they often end up in the sewage system as a result of excretion and disposal of unwanted quantities. Additionally, hormones from livestock waste can find its way into drinking water sources.⁸ There is evidence that hormones may not be effectively removed during wastewater treatment and as a result, significant amounts of these hormones may be present in drinking water sources.⁹ Both estrogens and androgens can be potent endocrine disruptors, perturbing normal hormone system functioning if individuals are exposed to these compounds from external sources.¹⁰ Concern over the potential for exposure prompted the development of EPA Method 539 to monitor the levels of the most commonly found hormones (Figure 1).¹¹ This method describes a procedure for quantifying hormones in drinking water using solid-phase extraction (SPE) followed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS).

In the experiments outlined here, Thermo Scientific™ Dionex™ SolEx™ SPE HRPHS cartridges were used to concentrate hormones that are present in a solution. These solvent extraction cartridges contain a polymeric stationary phase that is a neutral resin comprised of a 22 µm high-capacity, high-surface-area, divinylbenzene-based particle grafted with polyvinylpyrrolidone polymer. Its narrow size distribution yields high efficiency with low backpressure. This material has properties of a hydrophilic reversed-phase material and also has specific high capacity for polyphenolics and azo-containing substances. For more information on these cartridges, see the Dionex SolEx SPE cartridges Product Manual.¹²

Dionex SolEx SPE HRPHS cartridges are used on the Thermo Scientific™ Dionex™ AutoTrace™ 280 Solid-Phase Extraction instrument, a high-throughput workstation dedicated to automating SPE. It can process up to six samples in parallel, automatically conditioning the cartridge, loading large volume aqueous samples (20 mL to 4 L), rinsing, and then eluting with a choice of up to five reagents (Figure 2). See the Dionex AutoTrace 280 SPE Instrument Operator's Manual for additional information.¹³

This Technical Note describes the use of high-surface-area SPE (Dionex SolEx SPE HRPHS) to extract seven sex hormones from drinking water using the Dionex AutoTrace 280 SPE instrument. Extracts were subsequently quantified using high-performance liquid chromatography (HPLC) to determine the recovery.

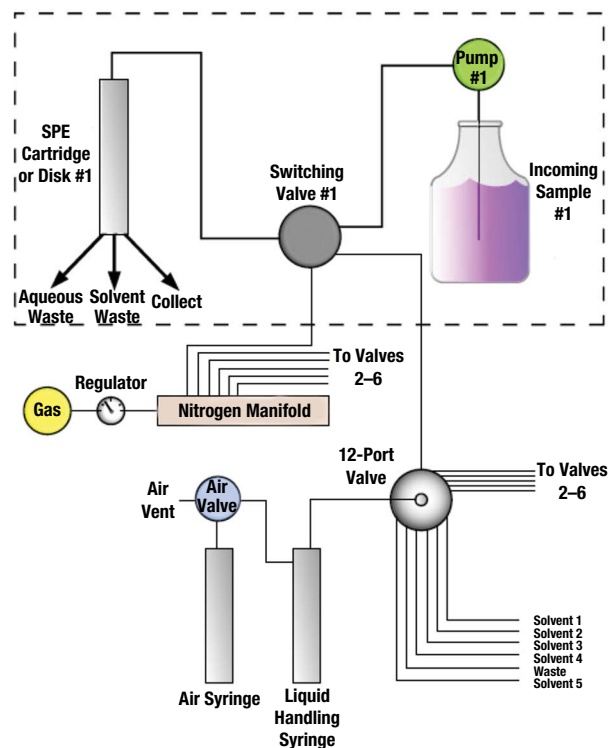


Figure 2. Dionex AutoTrace 280 SPE instrument fluid connections.¹³

Equipment

- Dionex AutoTrace 280 SPE instrument
- Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system:
 - DGP-3600M Dual-Gradient Micro Pump
 - WPS-3000TRS Rapid Separation Wellplate Sampler, Thermostatted
 - TCC-3000RS Rapid Separation Thermostatted Column Compartment
 - DAD-3000RS Rapid Separation Diode Array Detector (Without Flow Cell)
- Thermo Scientific™ Dionex™ Chromeleon™ 7.1 Chromatography Data System software

Reagents and Standards

- 18 MΩ-cm resistivity degassed deionized water
- Acetonitrile, Optima LC/MS (Fisher Scientific, P/N A955)
- Methanol, Optima LC/MS (Fisher Scientific, P/N A456)
- Testosterone C-III (Sigma-Aldrich™, P/N T1500)
- Estrone (Sigma-Aldrich, P/N E9750)
- Equilin (Sigma-Aldrich, P/N E8126)
- 17β-Estradiol (Sigma-Aldrich, P/N 250155)
- 17α-Ethinylestradiol (Sigma-Aldrich, P/N E4876)
- 4-Androstene-3,17-dione (CarboMer™, Inc, P/N 8-01510)
- 16α-Hydroxyestradiol (Estriol; Sigma-Aldrich, P/N E1253)

Dionex AutoTrace 280 SPE Instrument Conditions

Flow Rates	mL/min
Condition	15
Load	5
Rinse	20
Elute	5
Condition Air Push	15

SPE Parameters

Push Delay	5 sec
Air Factor	1
Autowash Volume	1 mL

Method

Step	Step Description	Volume	Solution
1	Condition Cartridge	10 mL	Methanol
2	Condition Cartridge	10 mL	Water
3	Dry Cartridge	5 min	Nitrogen Gas
4	Load	20 mL	Sample
5	Rinse	10 mL	Water
6	Elute	5 mL	Acetonitrile

To ensure that sample lines were filled and contained no air bubbles prior to starting, 10 mL of sample was loaded onto used cartridges, which were then rinsed with acetonitrile. Fresh cartridges were then placed into position prior to commencing the extraction experiments. Eluted solutions were collected into 15 mL conical tubes and then transferred to 1.5 mL glass vials for HPLC analysis.

HPLC Conditions

Columns:	Thermo Scientific™ Acclaim™ 120, C18, 5 µm (2.1 × 10mm), guard Thermo Scientific™ Acclaim™ 120 C18, 2.1 × 250 mm, analytical
Mobile Phase:	A. Water; B. Acetonitrile
Gradient:	10–55% B (0–4 min) 55% B (4–12 min) 100% B (12–16 min) 10% B (16–20 min)
Flow Rate:	0.2 mL/min
Inj. Volume:	2 µL
Temperature:	20 °C
Detection:	UV absorbance at 214, 240, and 254 nm
Noise:	< 0.1 mAu
System	
Backpressure:	1300–3800 psi (90–260 bar)

Table 1. Consumables list.

Product Name	Type	Part Number
Guard Cartridge	Acclaim 120, C18, 5 µm (2.1 × 10 mm)	069689
Analytical Column	Acclaim RSLC 120, C18, 2.2 µm (2.1 × 150 mm)	071399
Dionex Vial Kit, glass	1.5 mL vials with caps and septa, package of 100	055427
Dionex SolEx SPE HRPHS Cartridge	200 mg resin/6 mL cartridge, package of 48	088127
Conical Tubes	15 mL, case of 12	071056

Standard and Sample Preparation

Standard Preparation

Standard stock solutions of hormones at approximately 1000 mg/L were prepared by weighing ~10 mg of each into a 10 mL Erlenmeyer flask and then filling to the line with methanol. Solutions were sonicated for five min to mix. Calibration standards were prepared by adding the appropriate amount of stock solution to obtain the required final concentration.

Sample Preparation

Drinking water was fortified with 1 mg/L hormones and mixed by vortexing.

Results and Discussion

This Technical Note describes the extraction of hormones added to drinking water using Dionex SolEx SPE HRPHS cartridges, with the separation and quantification by HPLC. Because the goal of these experiments was to verify the reproducible recovery of the hormones present in solution by these cartridges, the sensitivity of HPLC at the concentrations used was sufficient for this determination, and mass spectrometry (as described in EPA Method 539) was not performed.

Chromatographic Separation Method

To achieve the optimal resolution for accurate quantification of the hormones analyzed, the column temperature was reduced from the typical 30 °C to 20 °C and the flow rate was reduced to 0.2 mL/min. An absorbance of 214 nm was chosen for run analysis due to the approximately equivalent signal response obtained for all hormones at this wavelength. As can be seen in chromatogram A of Figure 3, the hormone peaks had close to baseline resolutions (R_s , U.S. Pharmacopeia) at 50 mg/L, ranging from 1.4 (ethynylestradiol and estrone) to 2.9 (estriol). At hormone concentrations closer to that measured in fortified water samples and cartridge elutions (5 mg/L), the resolutions ranged from 1.6 to 3.7 (data not shown), all above the 1.5 minimum value for baseline resolution of similar sized peaks.¹⁴ Figure 3 also shows that only testosterone and androstenedione, structurally very similar molecules, showed substantial absorbance at 240 and 254 nm. While 54% acetonitrile was used here, for subsequent runs, 55% was used to shorten the run times, while achieving comparable peak resolution.

Column:	Acclaim RSLC 120 C18 column, 2.1 × 150 mm	Peaks:	R_s (USP)
Mobile Phase:	Water/Acetonitrile (CH ₃ CN)	1. Estriol	–
Gradient :	CH ₃ CN, 10–54% (0–4 min) 54% (4–12 min) 100% (12–16 min) 10% (16–20 min)	2. Estradiol	2.9
Flow Rate:	0.20 mL/min	3. Testosterone	1.7
Inj. Volume:	2 µL	4. Ethynylestradiol	1.4
Temperature:	20 °C	5. Equilin	1.7
Detection:	UV, A: 214 nm, B: 240 nm, C: 254 nm	6. Estrone	1.4
Sample:	Standard mix, 50 mg/L each	7. Androstenedione	–

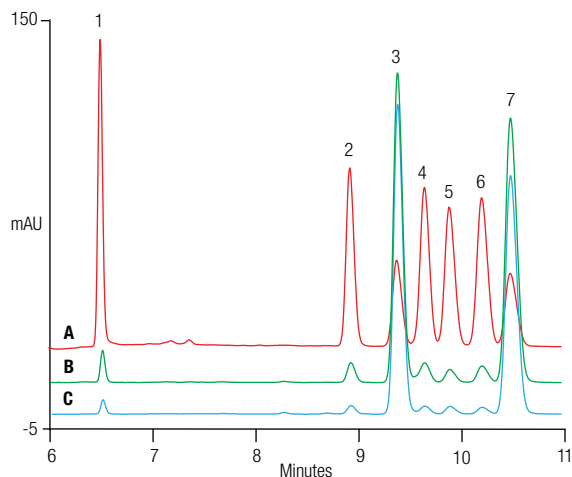


Figure 3. Hormone standards measured at various wavelengths following separation on an Acclaim RSLC 120, C18 column. The peak resolutions (R_s) are indicated.

Chromatographic Limits of Detection, Limits of Quantification, and Linearity

The limit of detection (LOD) was calculated as the concentration of hormone that gave a signal three times that of the noise, while the limit of quantification (LOQ) was the concentration that gave a signal-to-noise ratio of 10. As can be seen in Table 2, the LOD and LOQ ranged from 0.025 to 0.4 mg/L and 0.083 to 1.3 mg/L, respectively.

To determine linearity, triplicate injections of standards ranging from 0.05 to 10 mg/L were run. The coefficient of determination (r^2) was >0.999, indicating good fit of the standard curve to the data.

Table 2. LOD and LOQ for hormones at 214 nm.

Hormone	LOD (mg/L)	LOQ (mg/L)
Estriol	0.40	1.3
β-Estradiol	0.30	1.0
Testosterone	0.075	0.25
17α-Ethynylestradiol	0.030	0.10
Equilin	0.025	0.083
Estrone	0.030	0.10
4-Androstene-3,17-dione	0.050	0.17

Recovery

To determine the accuracy of the method, three aliquots of drinking water fortified with 1 mg/L of seven hormones were extracted in parallel and analyzed using HPLC. Figure 4 shows a comparison of the chromatograms for the fortified water prior to cartridge loading, a cartridge elution, and an injection of acetonitrile (used as the eluent for SPE) to show the background signal. When 20 mL of drinking water without added hormones was concentrated using SPE cartridges, the level of hormones present was less than the LOQ (data not shown). All of the cartridge elution peaks were well resolved, which facilitated quantification.

Column:	Acclaim RSLC 120 C18 column, 2.1 × 150 mm	Peaks: 1. Estriol
Mobile Phase:	Water/Acetonitrile (CH ₃ CN)	2. Estradiol
Gradient :	CH ₃ CN, 10–55% (0–4 min) 55% (4–12 min) 100% (12–16 min) 10% (16–20 min)	3. Testosterone
Flow Rate:	0.20 mL/min	4. Ethynylestradiol
Inj. Volume:	2 µL	5. Equilin
Temperature:	20 °C	6. Estrone
Detection:	UV, 214 nm	7. Androstenedione
Samples:	A. Cartridge elution B. Drinking water + 1 mg/L hormones C. Acetonitrile (eluent blank)	

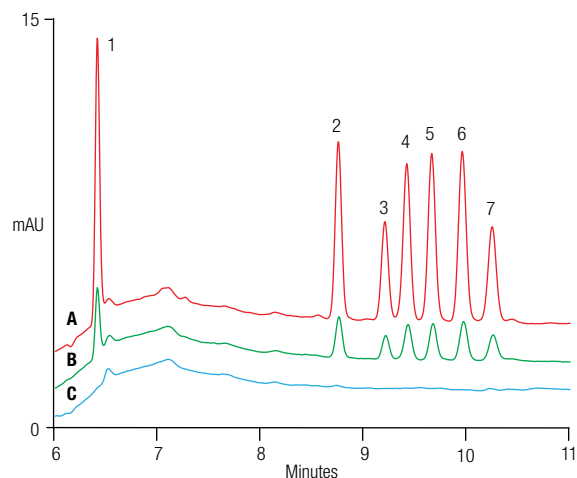


Figure 4. Chromatograms of hormones eluted from a Dionex SolEx SPE HRPFS cartridge (A), fortified drinking water prior to loading (B), and a blank injection (C).

Hormone	Fortified Water		Elutions								
			Cartridge 1			Cartridge 2			Cartridge 3		
	Average* (mg/L)	RSD	Average* (mg/L)	RSD	% Recovery	Average* (mg/L)	RSD	% Recovery	Average* (mg/L)	RSD	% Recovery
Estriol	1.04	1.8	3.90	2.1	93	3.97	2.7	95	4.53	2.0	109
β -Estradiol	0.96	2.8	3.91	2.7	102	3.92	2.5	102	4.51	2.0	118
Testosterone	1.05	1.2	3.98	1.4	95	4.01	2.1	95	4.56	1.5	108
17 α -Ethinylestradiol	0.92	2.9	3.85	1.6	104	3.96	2.7	107	4.57	2.2	124
Equilin	0.93	3.5	3.84	1.5	103	3.85	2.3	103	4.32	2.2	116
Estrone	0.96	3.2	3.88	2.3	101	3.90	2.6	101	4.45	2.1	115
4-Androstene-3,17-dione	1.07	4.4	3.82	1.9	90	3.82	2.0	90	4.35	1.5	102

*n = 5

The percent recovery from each cartridge was calculated by dividing the concentration determined for each hormone by the concentration factor achieved using the Dionex AutoTrace 280 SPE instrument (i.e., concentration factor of four; 20 mL loaded and 5 mL eluted). This number was then divided by the concentration of hormone present in the fortified drinking water solution. Table 3 shows the concentration of hormones loaded on the cartridges (Fortified Water) and the percent recovery from each of the three cartridges used. Five replicate HPLC runs were averaged for each elution. As can be seen, recovery of hormones from drinking water was approximately 100%, ranging from 90 to 124% with an RSD from 1.2 to 4.4. For EPA Method 539, average recoveries from 70 to 130% are acceptable.¹¹ While cartridges one and two had recoveries from 90 to 107%, cartridge three had values that were above 100% for all hormones (102–124%). This variability likely resulted from a small difference in the volume of eluent delivered during elution. The recovery values in all cases, however, are still within an acceptable range, indicating a robust method.

In the results presented, 20 mL aliquots of hormone-fortified water were analyzed. However, typically larger volumes are used for water analysis, which would permit

accurate quantification of hormones in drinking water that are at proportionately lower starting concentrations. With the Dionex AutoTrace 280 SPE instrument, up to 20 L can be automatically loaded onto each cartridge position, potentially increasing the sensitivity of this method 1000-fold compared to the quantities analyzed here.

Conclusion

The Dionex AutoTrace 280 SPE instrument greatly facilitates the analysis of aqueous samples that contain low-analyte concentrations by automating the process of concentrating them onto cartridges or discs containing absorbents with specific retention properties. Here baseline resolution of all of the hormones analyzed was demonstrated using the Dionex SolEx SPE HRPHS cartridges for sample extraction, followed by analysis on the UltiMate 3000 RSLC system using the Acclaim 120 C18 analytical column. Good recoveries from drinking water, ranging from 90% for androstenedione to 124% for ethinylestradiol, were shown. The recoveries achieved verify the suitability of these cartridges as a means to concentrate human hormones for subsequent quantification as per EPA Method 539.

References

1. The Associated Press. *An AP Investigation: Pharmaceuticals Found in Drinking Water*. http://hosted.ap.org/specials/interactives/pharmawater_site/day1_01.html (accessed Dec. 4, 2013)
2. World Health Organization (WHO), United Nations Environment Programme, Inter-Organization Programme for the Sound Management of Chemicals. State of the Science of Endocrine Disrupting Chemicals 2012: Summary for Decision-Makers. Bergman, A.; Heindel, J.J.; Jobling, S.; Kidd, K.A.; Zoeller, R.T. WHO Document Number WHO/HSE/PHE/IHE/2013.1.
3. European Parliament. Proposal for a Directive of the European Parliament and of the Council Amending Directives 2000/60/EC and 2008/105/EC as Regards Priority Substances in the Field of Water Policy. Procedure 2011/0429(COD). [www.europarl.europa.eu/oeil/popups/ficheprocedure.do?lang=en&reference=2011/0429\(COD\)](http://www.europarl.europa.eu/oeil/popups/ficheprocedure.do?lang=en&reference=2011/0429(COD)) (accessed Dec. 5, 2013)
4. U.S. Environmental Protection Agency (EPA). Basic Information about the Unregulated Contaminant Monitoring Rule 3 (UCMR3). <http://water.epa.gov/lawsregs/rulesregs/sdwa/ucmr/ucmr3/basicinformation.cfm#four> (accessed Oct. 18, 2013).
5. National Institutes of Health, National Institute on Aging. Hormones and Menopause: Tips from the National Institute on Aging. NIH Publication No. 09-7482. 2012.
6. Mayo Clinic. *Testosterone Therapy: Key to Male Vitality?* www.mayoclinic.com/health/testosterone-therapy/MC00030 (accessed Oct. 21, 2013).
7. Shelby, M.K.; Crouch, D.J.; Black, D.L.; Robert, T.A.; Heltsley, R. Screening Indicators of Dehydroepiandrosterone, Androstenedione, and Dihydrotestosterone Use: A Literature Review. *J. Anal. Toxicol.*, 2011 35(9), 638–655.
8. Wise, A.; O'Brien, K.; Woodruff, T. Are Oral Contraceptives a Significant Contributor to the Estrogenicity of Drinking Water? *Environ. Sci. Technol.*, 2011, 45(1), 51–60.
9. Silva, C.P.; Otero, M.; Esteves, V. Process for the Elimination of Estrogenic Steroids from Water: A Review. *Environ. Pollut.*, 2012, 165, 38–58.
10. National Institute of Environmental Health Sciences, Endocrine Disruptors webpage. www.niehs.nih.gov/health/topics/agents/endocrine/ (accessed Oct. 21, 2013).
11. Smith, G.A.; Zaffiro, A.D.; Zimmerman, M.L.; Munch, D.J. Method 539: Determination of Hormones in Drinking Water by Solid Phase Extraction (SPE) and Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS). Document No. 815-B-10-001. U.S. Environmental Protection Agency, Cincinnati, OH, 2010.
12. Thermo Fisher Scientific. Dionex SolEx Cartridges Product Manual. Thermo Scientific P/N 088175-02, Sunnyvale, CA, 2013. [Online] www.dionex.com/en-us/webdocs/115040-Man-SolEx-Cartridges-088175-02.pdf (accessed Jan. 6, 2014).
13. Thermo Fisher Scientific. Dionex AutoTrace 280 SPE Instrument Operator's Manual. Dionex Doc No. 065330, Sunnyvale, CA, 2012. [Online] www.dionex.com/en-us/webdocs/81032-Man-ASE-AutoTrace280-Operators-Oct2012-DOC065330-03.pdf (accessed Jan. 6, 2014).
14. Snyder, L.R.; Kirkland, J.J.; Dolan, J.W. Introduction to Modern Liquid Chromatography, 3rd ed.; Wiley and Sons: Hoboken, NJ, 2010.

www.thermofisher.com/chromatography

©2016 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific Inc. and its subsidiaries. This information is presented as an example of the capabilities of Thermo Fisher Scientific Inc. products. It is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details.

Africa +43 1 333 50 34 0
Australia +61 3 9757 4300
Austria +43 810 282 206
Belgium +32 53 73 42 41
Brazil +55 11 2730 3006
Canada +1 800 530 8447
China 800 810 5118 (free call domestic)
 400 650 5118

Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland +358 10 3292 200
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9494
Italy +39 02 950 591

Japan +81 120 753 670
Korea +82 2 3420 8600
Latin America +1 561 688 8700
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
New Zealand +64 9 980 6700
Norway +46 8 556 468 00

Russia/CIS +43 1 333 50 34 0
Singapore +65 6499 9999
Sweden +46 8 556 468 00
Switzerland +41 61 716 77 00
Taiwan +886 2 8751 6655
UK/Ireland +44 1442 233555
USA +1 800 532 4752

TN70898-EN 07/16S

Thermo
 S C I E N T I F I C

Part of Thermo Fisher Scientific