Comparison of ergovaline determinations between the laboratories in the United States and Japan

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Abstract

Quality assurance and quality control is the foundation of any diagnostic test. The two laboratories in the United States that use HPLC to quantitate endophyte toxins in tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*) are Oregon State University (OSU) and University of Missouri (MU). Japan, the major importer of grass straw has six new laboratories that will test agricultural imports for endophyte toxins. A quality assurance program was set up between the Japanese Ministry of Agriculture and the State of Oregon. The latter includes both OSU and the MU. All units are using an accurate crystalline standard and have exchanged "check" samples among themselves. To date OSU and MU have values that differ by 10%. OSU has identified a contaminating and coeluting peak as the cause of the differences. Both laboratories are changing to a Gemini column to rectify the differences. Japanese laboratories are in the process of evaluating their split check samples.

Keywords: quality assurance, quality control, endophyte, tall fescue, perennial ryegrass

Introduction

Quality assurance and quality control are the foundation of any diagnostic test. In the United States there are primarily two laboratories that use high performance liquid chromatography (HPLC) to quantitate endophyte toxins in tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*); they are Oregon State University (OSU) and University of Missouri (MU). Japan, Korea, and Taiwan have become the major importers of these grass straws, especially perennial ryegrass and have been concerned over the rise in endophyte toxin levels in the grass straw relative to animal health and safety.

On May 29, 2006, Japan's Ministry of Agriculture, Forestry and Fisheries in accordance with their Ministry of Health, Labor and Welfare instituted a "positive list" system to prohibit the distribution of foods that contain agricultural chemicals above a certain level of maximum residue limits (MRLs). Japan has six new laboratories that will test agricultural imports for toxin levels as well as pesticides, herbicides and veterinary drugs. A laboratory has been established at OSU by the United States exporters to insure that the shipments of grass straw contain endophyte alkaloid levels that are below the levels known to cause clinical disease (threshold levels). Thus, it is imperative that the assays for these respective alkaloid toxins give consistent values between Japan and the United States.

A quality assurance programme was set up between the Japanese Ministry of Agriculture and the State of Oregon. The latter includes both the MU Veterinary Medicine Diagnostic Laboratory and the OSU College of Veterinary Medicine. All three of these units have insured that they are using an accurate crystalline standard. Moreover, they have exchanged "check" samples among themselves. The State of Oregon will be an independent examiner and send out periodic "check samples" and compare results among the participants.

Materials and Methods

Reference material, ergovaline tartrate, was prepared by Dr. Forrest Smith, Department of Pharmacal Sciences, Auburn University, Auburn, AL. Chemical analysis and assessment of purity of the crystalline standard utilised NMR, CHN, and HPLC/MS (Nuclear Magnetic Resonance, Carbon Hydrogen Nitrogen, and HPLC/Mass Spectrometry).

Preparation of quality control seed material

Bags of tall fescue seed, L65, SR8500, and SR8550 were transported from dry storage to USDA-ARS NFSPRC, Corvallis, OR, USA. Using a seed sorter each bag was emptied and mixed to assure uniformity of the seed from each bag. Four kg aliquots were prepared using mixtures of the seed lines according to the following chart:

L65	4 kg	2 kg	0	3.35 kg	2.65 kg
SR8500	0	2 kg	4 kg	0	0
SR8550	0	0	0	0.65 kg	1.35 kg

The 4 kg mixtures were then sent through the seed sorter 25 times to assure uniformity. The 4 kg aliquots were then split into 1 kg aliquots using the seed sorter. The 1 kg samples were double bagged into brown paper bags for storage at ambient temperature.

Preparation of quality control straw material

One bale of tall fescue straw was chosen from two separate lots of straw testing at 400 and 1000 ppb ergovaline. Grab samples from each bale were ground in a mill (Tecator, H ̄gan≅s, Sweden) to pass through a 0.5 mm screen and identified as #400 and #1000. The final ground weight of the two samples was 4 kg each. The individual samples were mixed in a plastic zip-lock storage bag by hand for 30 minutes and then frozen at -20˚C. After remixing, 50 g aliquots were removed from these bags for ergovaline analysis.

OSU short method

The procedure was adapted from a previously described method (Craig *et al*. 1994).

Reagents HPLC grade acetonitrile, methanol, and chloroform and reagent grade ammonium carbonate, sodium hydroxide, and ergotamine tartrate were purchased from commercial sources (J.T. Baker, Phillipsburg, NJ and Sigma-Aldrich, St. Louis, MO).

Solutions Ergotamine tartrate solution at 1 µg/ml in chloroform was used as the internal standard and prepared daily. A 1 mM sodium hydroxide solution in water and a chloroform/acetone (75/25, v/v) wash solution were also prepared. The mobile phase for the HPLC analysis consisted of acetonitrile/2.6 mM ammonium carbonate (70/30 v/v).

Instrumentation Analyses were performed by HPLC with fluorescence detection. An isocratic HPLC pump (PerkinElmer, Shelton, CT) was set at a flow rate of 1 ml/min and an autosampler (PerkinElmer, Shelton, CT) with a 200 µl sample loop was set to

Table 1 Comparison of methods used for ergovaline analysis at the University of Missouri (MU) and Oregon State University (OSU).

Step	OSU	MU
Sample preparation	1.0 g dried plant material ground to pass through 0.5 mm screen in 15 ml screw top test tube with Teflon lined cap.	1.0 g dried plant material in a polypropylene screw cap bottle.
Additions to sample	9 ml chloroform, 1 ml 10.0 µg/ml ergotamine tartrate in chloroform (internal standard), and 1 ml 1.0 mM sodium hydroxide.	40 ml chloroform/ 0.01 M sodium hydroxide (9:1), and 1.0 ml methanol containing 940 ng/ml ergotamine tartrate.
Mixing time	Shake or rotorack for 18-24 hours.	Shake for 30 + 5 min.
Separate supernatant from particulate	Centrifuge at 2000 RPM for 5 min.	Filter thru Whatman PS-1 filter paper.
Ergosil SPE column conditioning	5 ml chloroform	2 ml cholroform
Supernatant	5 ml	20 ml
Wash	1 ml chloroform:acetone (75:25) 1 ml chloroform:acetone (75:25) 1.5 ml methanol	5 ml acetone:chloroform (75:25) 1 ml ethyl ether
Elute	2.5 ml methanol	Methanol to 2 ml final volume and place in HPLC vials
Concentration	Dry under nitrogen at 50˚ C	
Reconstitute	0.5 ml methanol and place into HPLC vials	
HPLC analysis mobile phase	70% acetonitrile 30% 0.2 mg/l ammonium carbonate. Flow rate is 1 ml/min.	62% water 38% acetonitrile with 200 mg/L ammonium carbonate. Flow rate is 1 ml/min
Column	Jordi RP SM-500, 5µ, 500Å (Alltech #105500), guard column 7.5X4.6 mm (Alltech #1005595)	Luna C18, 150 X 4.6 mm, 3µ, (Phenomenex)
Injection volume	20 µl	20 µl
Fluorescence detector	Excitation 250 nm, emission 420 nm	Excitation 250 nm, emission 420 nm
Standards	Standard reference material validated using ergovaline tartrate purchased from Dr. Forrest Smith	Ergovaline tartrate purchased from Dr. Forrest Smith

inject a sample volume of 20 µl. The ergopeptine alkaloids were visualised using a fluorescence detector (PerkinElmer, Shelton, CT) set at an excitation wavelength of 250 nm and an emission wavelength of 420 nm. Data collection was via an interface to a PC-based data system (PerkinElmer, Shelton, CT). An analytical column packed with divinyl benzene (Alltech, Deerfield, IL) 5-µm particle size was used in conjunction with a guard column cartridge (Alltech, Deerfield, IL) with similar packing.

Extraction Seed and straw samples were ground in a mill (Tecator, H ̄gan≅s, Sweden) to pass through a 0.5 mm screen. 1.0 g of the ground plant material was weighed out into a 16 x 125 mm glass screw top tube. To each tube of sample, control or reference material, 10 ml of chloroform plus 1 ml of the internal standard (ergotamine) and 1 ml 0.001 N NaOH was added. The tubes were capped and mixed 18-24 h in the dark. The tubes were then centrifuged at 1,700 *g* and 5 ml of the supernatant was added to a conditioned Ergosil (Alantech, Newark, DE) SPE column. The column was washed twice with wash solution and with 1 ml of methanol. The sample was then eluted with 2.5 ml methanol and the eluant was collected in a 12 x 75 mm test tube. The tube was dried under nitrogen and reconstituted with 0.5 ml methanol. After sonication and mixing the extract was transferred to amber 12 x 32-mm HPLC vial and sealed for analysis.

Quantitation Ergovaline peak elution time was at 4 min and the ergotamine peak elution time was at 6 min with a total run time of 12 min. Using the data system, Perkin Elmer TotalChrom, a

Table 2 Comparison of values of ergovaline in five seed samples as determined by OSU and MU.

Seed Identification	OSU, ergovaline (ppb)	MU, ergovaline (ppb)
L65	<100 (n=50)	8 ± 1.30 (n=5)
L65/SR8500 (1:1)	145 ± 18 (n=51)	105 ± 8 (n=10)
SR8500	279 ± 33 (n=50)	232 ± 63 (n=14)
L65/SR8550 (21:4)	472 ± 49 (n=88)	482 ± 77 (n=10)
L65/SR8550 (33:17)	981 ± 91 (n=54)	1001 ± 153 (n=10)

Table 3 Comparison of a high and low ergovaline containing straw by OSU and MU. laboratories.

Straw Identification	OSU, ergovaline (ppb)	MU, ergovaline (ppb)
Straw #400	358 ± 19 (n=18)	308 ± 28 (n=16)
Straw #1000	859 ± 45 (n=10)	695 ± 78 (n=9)

Table 4 Comparison of the results of a check seed sample run by both OSU and MU.

	Oregon State University	University of Missouri
Day to day variation (ppb)	316 ± 18 (n=204)	269 ± 16 (n=6)
Within day variation (ppb)	311 ± 12 (n=6)	//
LOD* (ppb)	31	//
LOQ** (ppb)	100	50
% Recovery	91	//

* Limits of detection
** Limits of quantitation

standard curve was constructed from a linear regression fit of the peak height versus the amount of analyte injected.

OSU long method

The list of reagents, solutions, instrumentation, and extraction were the same as the above method with the exception of the HPLC column and mobile phase. The HPLC column was a bonded and end capped $C18^{j}$ with a C18 guard column (Phenomenex, Torrance, CA). A gradient programme was run at a flow rate of 1.0 ml per minute with 2.1 mM ammonium carbonate as mobile phase A and acetonitrile as mobile phase B as follows: start 80%A:20%B; 0-20 min a linear change to 30%A:70%B; 20-25 min 30%A:70%B; 25-30 min a linear change to 80%A:20%B; 30-35 min 80%A:20%B. The ergovaline/ergotamine peak elution times were at 14.8 min and 17 min respectively.

MU short method

The procedure was adapted from previously described methods (Rottinghaus *et al.* 1991 and Hill *et al.* 1993).

Extraction and cleanup A 1 g finely ground sample was weighed into a widemouth polypropylene screw-cap bottle with 40 ml chloroform:0.01 M sodium hydroxide (9:1) to which 1 ml of methanol containing 940 ng/ml ergotamine tartrate had been added as an internal standard. The mixture was placed on a wrist-action shaker (Burrel Corp., Pittsburgh, PA) for 30 min. The contents were allowed to settle, and a 25-ml aliquot of chloroform was filtered (Whatman International, Maidstone, England) to remove any residual water. The ergot cleanup column contains silica gel and an organic binder which tightly binds ergopeptine alkaloids. The column consists of a 12.7-mm biological disc (Schleicher & Schuell, Fisher Scientific Products, St. Louis, MO) placed in a 6-ml disposable syringe barrel; 0.5 g of ground Ergosil silica gel (Analtech, Newark, DE) was added, followed by another biological disc, 1 g sodium sulfate, and another biological disc. The column was prewashed with 2 ml chloroform, and 20 ml filtrate was applied. Pigments were removed from the silica gel by washing the column with 5 ml acetone:chloroform (74:25), followed by 1 ml ethyl ether. Vacuum was continually applied until the column was no longer cool to the touch, indicating that ether was totally removed. Ergopeptine alkaloids were eluted with 2 ml methanol and combined with 2 ml distilled water. The eluant was passed through a 0.22-µm nylon 66 membrane filter (MSI, Westboro, MA) prior to HPLC analysis.

Analytical methodology Ergopeptine alkaloids were determined by HPLC with florescence detection using a previously described procedure (Scott 1980). Twenty µl of eluant was injected into an HPLC fluorescence detector (excitation 250 nm, emission 420 nm) and an 8.5-cm C18 (3 µm) analytical $column^{r}$. The mobile phase, 38% acetonitrile in a 200-mg/L solution of ammonium carbonate in distilled water, was pumped at 1 ml/min. The elution time was 15 minutes.

Japanese method

Ergovaline was extracted from tall fescue and perennial ryegrass straw with a 20% acetic acid. The extract was spiked with ergotamine as an internal standard and filtered and purified using

Figure1 NMR of ergovaline.

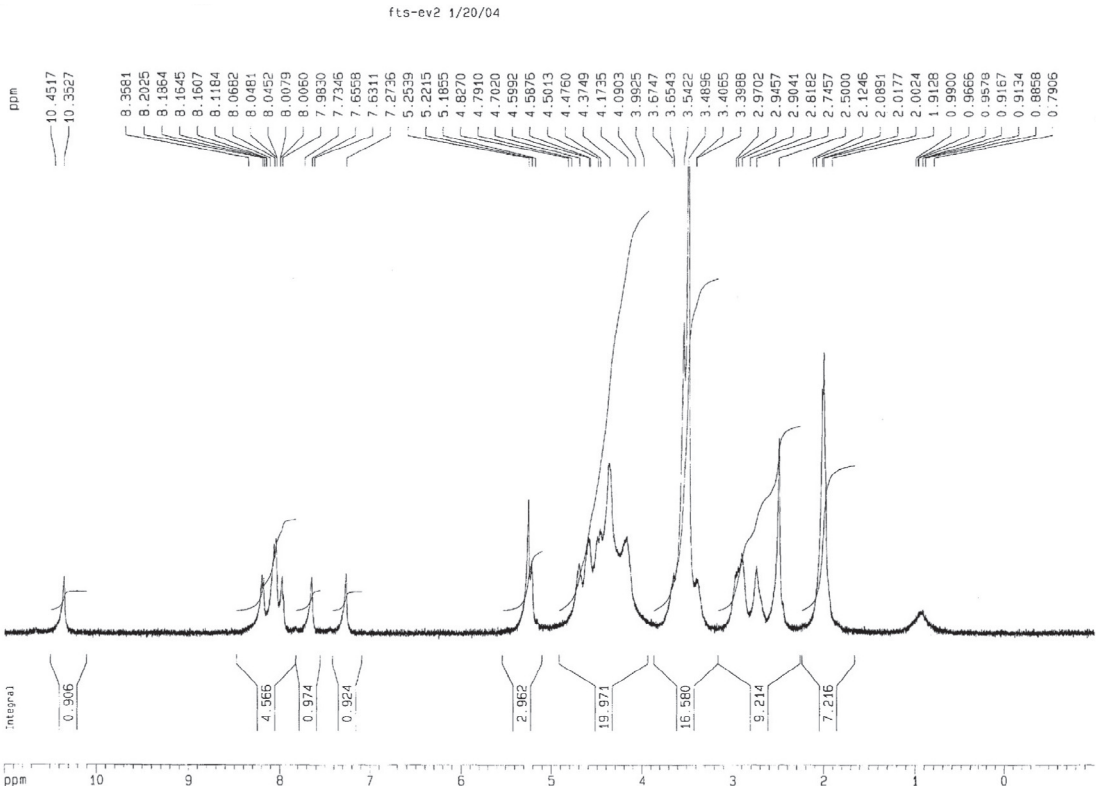


Figure 2 CHN of ergovaline.

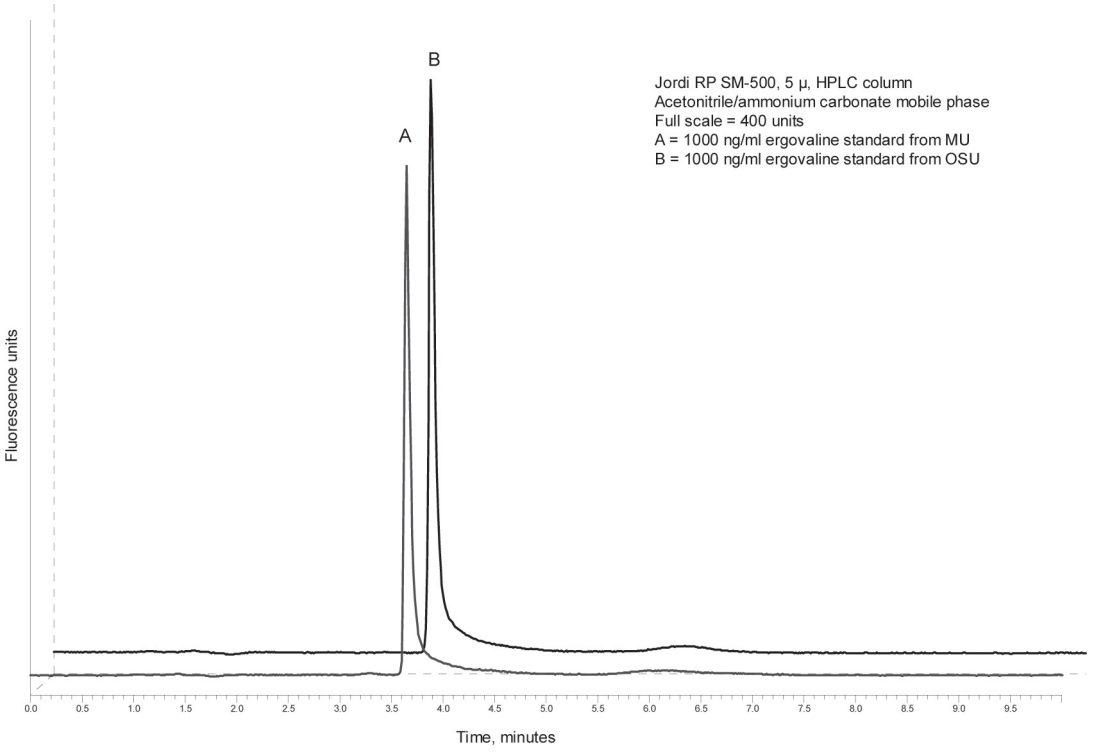
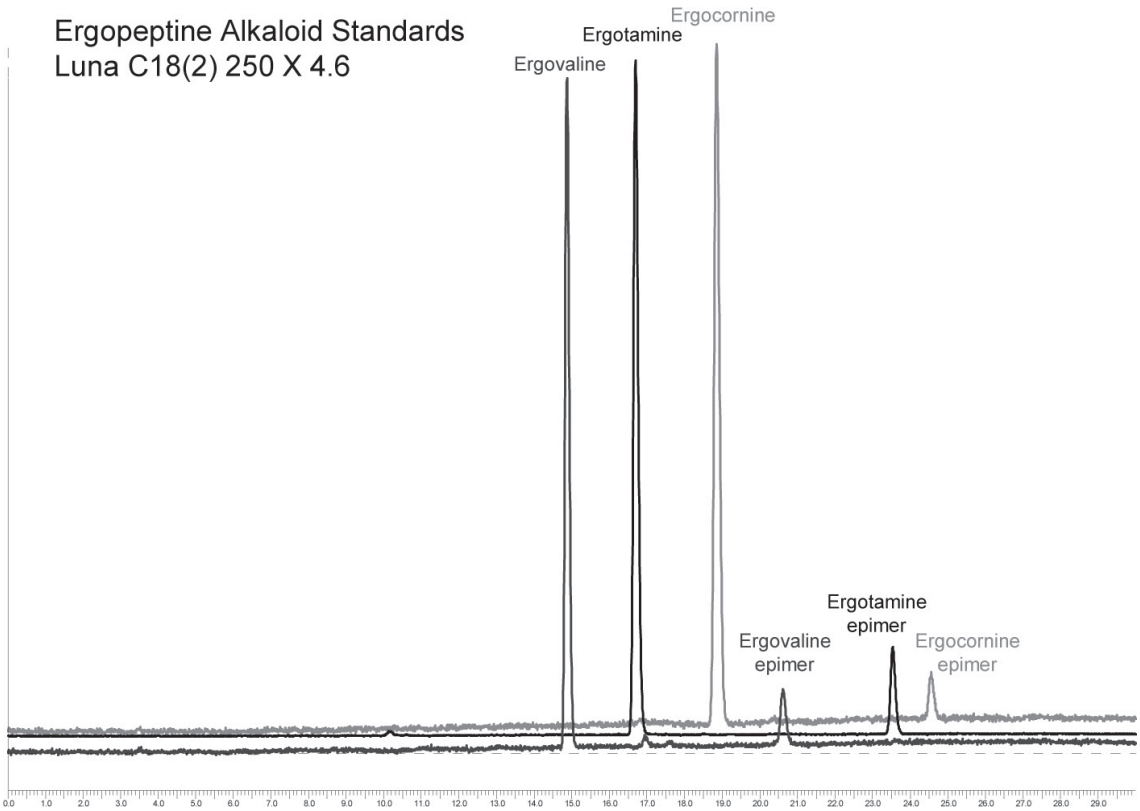


Figure 3 Ergopeptine alkaloid standards.



a Sep-Pak Plus C18 SPE column. The ergovaline and ergotamine were separated on a Capcell PAK C18 HPLC column with water-methanol-acetonitrile-ammmunium hydroxide (45:45:10:0.15) as the mobile phase and detected by a fluorescence detector (excitation 315 nm, emission 415 nm) (Saiga *et al*. 2003).

Results

Table 1 documents the protocols for both OSU and the UM. Both use a solvent/solvent extraction with a SPE clean-up step and involve 14 steps in the analysis. Table 2 is the comparison of values of ergovaline in five seed samples as determined by OSU and UM. Note the 10% difference. Both authors feel the difference is due to undefined coeluting peaks. The values are, however, well within the range of accuracy. Table 3 is the comparison of a high and low ergovaline values in straw from both OSU and UM laboratories. Again, note the 10 to 15% difference between the laboratories. Table 4 is the comparison of the results for day-to-day variation and within day variation of both OSU and UM using matching seed samples. The limits of detection and the limits of quantitation are provided in the table.

Figure 1 is the NMR of the crystalline standard verifying its purity. Figure 2 is the comparison of ergovaline standards from UM and from OSU. This represents the same crystalline material run in both universities. Figure 3 is an HPLC/MS chromatogram of the ergopeptine alkaloid standards and their epimers purchased commercially[b]. Those standards demonstrate elution times for three of the most common ergopeptine alkaloid toxins and can be used to compare to material from clinical cases.

Discussion

The ergovaline assay measures values in parts per billion (ppb) as opposed to the many that analyse for parts per million (ppm). Moreover, it is a complicated assay with 14 extraction and clean-up steps in the protocol. The results from the MU and OSU are comparable especially at the higher concentrations. However, values are consistently approximately 10% lower at the MU compared to OSU. The difference in concentrations of ergovaline was due to a consistent contaminating coeluting peak that accounted for approximately 70 ppb of the difference. As a consequence, a new Gemini column has been put into use by both laboratories which separates the coeluting peak from the ergovaline peak. The Japanese laboratories are still evaluating their split samples.

Central to this quality assurance/quality control program is having a pure and stable standard and having it utilised by all the US and Japanese laboratories. This has been a confounding issue in the Japanese analyses and in early 2007 a new standard will be shared between all the laboratories which should resolve any differences now seen.

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SOURCES AND MANUFACTURERS

a. J.T. Baker, Phillipsburg, NJ.
b. Sigma-Aldrich, St. Louis, MO.
c. Series 200 Quartnary Pump, PerkinElmer, Shelton, CT.
d. Series 200 Autosampler, PerkinElmer, Shelton, CT.
e. LS40 Fluorescence detector, PerkinElmer, Shelton, CT.
f. 900 Series Interface and TotalChrom Workstation, PerkinElmer Instruments, Shelton, CT.
g. Jordi RP-DVB 500D 5µ 4.6 x 150mm, Alltech, Deerfield, IL.
h. Jordi RP-DVB 4.6 x 7.5mm, Alltech, Deerfield, IL.
i. Cyclotec 1093 sample mill, Tecator, H ̄gan≅s, Sweden.
j. Luna C18(2), 5µ, 4.6 x 250mm, Phenomenex, Torrance, CA.
k. Security C18 guard column, Phenomenex, Torrance, CA.
l. Burrel Corp. Pittsburgh, PA.
m. Whatman PS-1 Filter paper, Whatman International, Maidstone, England.
n. 740-E, Schleicher & Schuell, Fisher Scientific Products, St. Louis, MO.
o. Monoject, 6 ml disposable syringe barrel, Division of Sherwood Medical.
p. Analtech Ergosil, Newark, DE.
q. MSI, Westboro, MA.
r. Model 250 LC Binary Pump, Model LS-4 Fluorescence Spectrometer, and Pecosphere 8.5 Cartridge C18 Analytical HPLC Column (3 µm), Perkin Elmer, Norwalk, CT.

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