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Detection of lysergic acid in ruminal fluid, urine, and in endophyte-infected tall fescue using high-performance liquid chromatography

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Abstract. Ergot alkaloids present in endophyte-infected tall fescue induce fescue toxicosis in livestock consuming the plant. The lysergic acid (LA) ring structure is a common moiety among the ergot alkaloids. Little is known about the bioavailability of LA because of limitations in available analytical protocols. Thus, a high-performance liquid chromatography procedure was developed to analyze biological matrices for LA. The biological matrices of interest were tall fescue straw and seed, and ruminant feces, urine, and ruminal fluid. Lysergic acid was added to each matrix at a high (150 ng/ml) or low (30 ng/ml) level. Using the high-level addition, the greatest recovery of LA was obtained from ruminal fluid, feces, and urine ($P < 0.05$), with an average 85.1% recovered. At the low level, a greater recovery of added LA was observed in the ruminal fluid, urine, and feces (82.1%; $P < 0.05$) than that in the other 2 matrices (62.6%). The limit of quantitation (LOQ) in ruminal fluid and urine was 5.5 and 18.4 ng/ml, respectively. Seed, straw, and feces had higher LOQ (24.2, 14.5, and 36.0 ng/g, respectively). Limit of detection (LOD) was 1.64, 10.80, 4.35, 5.52, and 7.26 ng/g for ruminal fluid, feces, urine, seed, and straw, respectively. To test the assay in vivo, samples of ruminal fluid and urine were collected from steers consuming a diet containing 400 ng of ergovaline/g and 30 ng of LA/g. All matrices sampled resulted in levels above the LOD and LOQ for the assay, indicating that this assay is sufficiently sensitive for use in assessing the bioavailability of LA.

Key words: Cattle; ergot alkaloids; high-performance liquid chromatography; lysergic acid; rumen; tall fescue.

Introduction

It has been proposed that the ergot alkaloids, ergovaline or lysergic acid (LA; Fig. 1), or both, are the toxic entities causing fescue toxicosis.[3,7] Ergovaline is usually present in the highest concentration in endophyte-infected tall fescue and, therefore, is the ergot alkaloid most commonly implicated in the onset of fescue toxicosis. Ergot alkaloids are potent vasoconstrictors that induce the loss of extremities in cold weather and increased time spent in the shade during hot weather, leading to decreased average daily gain.[4] One of the biggest challenges in the prevention of fescue toxicosis is the limited analytical capability to assess the presence and metabolism of LA in mammalian systems.

Currently, the predominant method in diagnostic laboratories for determining ergovaline content in tall fescue plant tissues is high-performance liquid chromatography (HPLC).[1,5] However, this method is specific for ergovaline and does not quantify simpler ergoline alkaloids (LA, lysergic acid amide, ergonovine).[6] Therefore, an enzyme-linked immunosorbent assay (ELISA) was developed by Hill and coworkers[2] to assay for total ergot alkaloid concentration in a variety of matrices. However, the ELISA had variable specificity to individual ergot alkaloids, with 50% binding efficiency at 10^{-7} mol/liter for ergonovine and at 10^{-8} mol/liter for LA. Ergot alkaloids with larger groups (ergotamine tartrate, ergocryptine, ergocornine, and ergocristine) did not exhibit strong binding to the monoclonal antibodies used in the ELISA.[6] Additionally, significant within-run and day-to-day variation was observed,[2,6] suggesting that the method was not robust. Assays using HPLC improve method selectivity; therefore, the objective of the study reported here was to develop an HPLC assay for LA quantification.

Materials and methods

Animals. Ruminal fluid, feces, and urine were collected from 4 steers (650 to 730 kg) fitted with ruminal cannulas. Each animal received both treatments, with adequate adaptation time between treatments (>28 days) before sampling. Animals were allowed ad libitum access to water and a diet of endophyte-infected tall fescue straw (E+) or endophyte-free tall fescue straw (E−). The E+ straw diet

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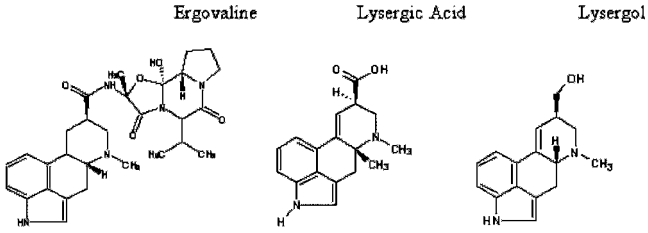

Figure 1. Chemical structure of ergovaline, lysergic acid, and lysergol.

had an ergovaline concentration of 400 ng/g, and the E− straw diet had an ergovaline content <10 ng/g determined by the method of Craig et al.[1] Ergovaline content was analyzed to account for sources of LA in the diet. Multiple samples of each matrix (5 to 6 samples/animal) were collected from animals fed E+ diets; additional samples (>2×) of each matrix were collected from animals fed E− diets to provide blank matrix samples for validation of the method. All procedures involving sampling from animals were approved by the Oregon State University Institutional Animal Care and Use Committee (IACUC No. 2813).

Ruminal fluid. Ruminal fluid was collected via suction strainer[a] from animals consuming an E+ or E− straw diet as described previously. Approximately 20 ml was collected, placed immediately on ice, transported to the laboratory, and stored at −20°C until analysis. Ruminal fluid was divided into duplicate aliquots of 6.5 ml and was concentrated, using a Savant ISS-100 centrifugal evaporator[b] set at high temperature (approx. 65°C), with an evaporation time of approximately 5 hr. This was done to concentrate the LA in the ruminal fluid to minimize the amount of sample processed through the solid-phase extraction (SPE) column. Volatility of LA at differing evaporation temperatures was evaluated, and evaporation at approximately 65°C did not affect recovery of LA. The concentrated ruminal fluid was resuspended in 3 ml of water from an ion-exchange reverse osmosis membrane filtration system (18.2 megaohm-cm water quality, hereafter referred to as pure water) and pH was adjusted to between 5.0 and 5.5 with 10% acetic acid.[c] The resulting solution was centrifuged at 2,000 × *g*[d] for 10 min. The supernatant was transferred for extraction and purification of LA.

Urine. Urine was collected in amber pill vials and was stored at −20°C until analyzed. A 3.0-ml aliquot was adjusted to a pH between 5.0 and 5.5 with 10% acetic acid. Acidified samples were centrifuged at 2,000 × *g* for 10 min, and supernatant was transferred for LA extraction and purification.

Analysis of straw and seed samples. Approximately 1 g of straw or seed ground to fit through a 0.5-mm screen was weighed into a 16 × 125-mm, glass, screw-cap, test tube. To the tube was added 10 ml of pure water : acetonitrile[e] (1 : 1). Tubes were sealed with Teflon-lined caps and rotated on a hematology/chemistry mixer[f] for at least 16 hr in the dark at room temperature. The mixture was separated by centrifugation for 10 min at 2,000 × *g*. A 5-ml aliquot of the liquid layer was adjusted to pH between 5.0 and 5.5 with 10% acetic acid for LA extraction and purification.

Stability of lysergic acid. The stability of LA in the solvent used to extract the straw, seed, and feces was tested by adding 150 mg of LA/ml into the extraction solvent. A time series sampling was done, with samples withdrawn at 0, 6, 18, and 48 hr after addition of the LA. Samples were transferred, and pH was adjusted to between 5.0 and 5.5 with 10% acetic acid for LA extraction and purification.

Standard preparation. Standards were prepared by adding 1.0 ml of methanol to 0.1 mg of crystal lysergic acid[g] (100,000 ng/ml); this solution was used to prepare a stock solution of 2,700 ng of LA/ml in 50 : 50 methanol : 0.05 M phosphate buffer,[h] pH 8.5. A serial dilution of standard concentrations was made using Hamilton syringes for accurate measurement. The pH of the resulting solutions was adjusted to between 5.0 and 5.5 with 10% acetic acid and was subjected to the same LA extraction and purification as were samples.

Extraction and purification of LA. Lysergic acid was extracted and purified from the resulting supernatant from all matrices using strong cation-exchange, SPE cartridges[i] on a vacuum manifold.[j] The SPE cartridge was preconditioned with 3 ml of HPLC-grade methanol[e] followed by 3 ml of 0.1 M HCl[e] and two 3-ml portions of pure water. The SPE cartridge was not allowed to dry between each addition. Preconditioning eluents were discarded. The acidified supernatant was loaded onto the SPE cartridge followed by two 3 ml portions of pure water (pH 6.8). Lysergic acid was eluted from the SPE cartridge with 3 ml of methanol : ammonium hydroxide[k] (95 : 5). The LA-containing fraction was collected and concentrated to dryness using a centrifugal evaporator at room temperature (Savant ISS-100[b]). The residue was reconstituted in 200 µl of 50 : 50 methanol : 0.05 M phosphate,[h] pH 8.5. Reconstituted samples were placed in an ultrasonic bath[l] for 30 sec, then were transferred to a 1.7-ml microcentrifuge tube with a centrifuge filter.[m] Samples were centrifuged at 10,000 × *g* in a microcentrifuge[n] for 5 min. The filtrate was transferred to HPLC vials equipped with a 150-µl glass polyspring insert.[o] The extraction and purification was performed immediately after acidification and took approximately 15 min/sample. All samples were analyzed by HPLC the day of extraction. Time of storage and LA recovery from stored extracted samples was not tested.

Instrumentation. The HPLC system consisted of a solvent delivery module[p] equipped with a sample injector[q] and a fluorescence detector,[r] with excitation wavelength at 250 nm and emission wavelength at 420 nm.

Liquid chromatography. Analysis by HPLC was carried out using a guard column hand-packed with Pellicular C18 material[j] and a Luna C18(2) analytical column[s] (150 × 3.0 mm id, 5-µm particle size) eluting at 1 ml/min under isocratic conditions with a 94-to-6 ratio of 0.05 M phosphate buffer (pH 8.5) and acetonitrile.

Accuracy and precision. Accuracy was tested by adding known concentrations of LA into the biological matrix of interest prior to extraction and purification of LA. Urine, ruminal fluid, and feces collected from steers consuming the

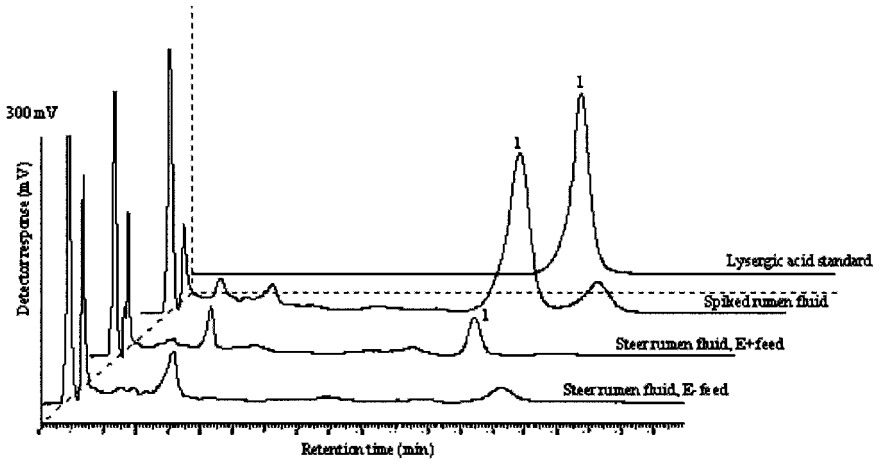

Figure 2. Overlay of spiked ruminal fluid samples and lysergic acid found in ruminal fluid samples collected from steers consuming endophyte-infected and endophyte-free tall fescue straw. [1] Lysergic acid peak in 270 ng of lysergic acid standard/ml, whole ruminal fluid 300 ng/ml spike, or whole ruminal fluid from steers consuming endophyte-infected straw (400 ng of ergovaline/g).

E− straw diet, as well as endophyte-free seed and straw, had pure LA—30 (low) or 150 ng/g (high)—added to the sample. This procedure was replicated 3 times using separately prepared samples on 3 days. Each sample was injected in duplicate and compared with the known amount added. Precision was tested by evaluating the reliability of injection by preparing a standard solution of LA in each matrix and injecting the solution 9 times. Standard deviation for each run was calculated, with SD $\leq$ 10% considered acceptable. Coefficient of variation (CV) was calculated as the SD divided by the mean. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to previously published methods.[8]

Statistical analysis. Data were analyzed as a completely randomized design using the generalized linear models (GLM) procedure.[t] The model includes effects of amount of LA added, recovery of added LA, day of analysis, matrix, and day $\times$ matrix interactions; least squares means were separated using PDIFF[t]. Differences with P-value $<$ 0.05 were considered significant.

Results

Chromatographic conditions were evaluated using purified LA. Figure 2 shows representative chromatograms of LA standard, spiked ruminal fluid, and ruminal fluid collected from steers consuming E+ and E− straw diets. Results in Figure 2 illustrate that this method is sufficiently sensitive to detect LA in ruminal fluid of animals consuming the E+ straw diet with 400 ng of ergovaline/g. Additionally, LA was not detected in the ruminal fluid of steers fed the E− diet. Chromatographs of LA detection in urine (Fig. 3) indicate that there are more peaks prior to the LA peak, but none of which appears to interfere with LA detection. This observation is supported by data in Tables 1 and 2 showing >80% recovery and precision of the assay over time.

Recovery of LA (Table 1) from feed, ruminal fluid, feces, and urine matrices that is important to

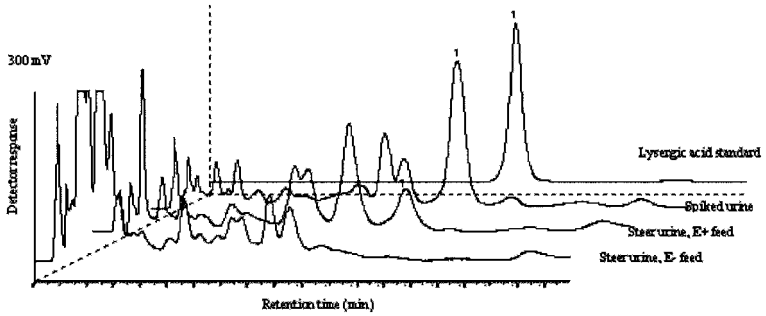

Figure 3. Overlay of spiked urine samples and lysergic acid found in urine samples collected from steers consuming endophyte-infected or endophyte-free straw. [1] Lysergic acid peak in 270 ng of lysergic acid standard/ml, urine 300 ng/ml spike, or urine collected from steers consuming endophyte-infected tall fescue straw or endophyte-free tall fescue straw.

Table 1. Precision evaluation of lysergic acid recovery when added to ruminal fluid, urine, feces, tall fescue straw, and tall fescue seed at high and low levels.

Matrix	Lysergic acid added (ng/g)	Lysergic acid detected (ng/g)*	Recovery (%)	CV (%)†
Bovine whole ruminal fluid	150	121.2 ± 7.8	80.8	6.5
	30	24.1 ± 0.5	80.3	2.3
Bovine urine	150	131.9 ± 9.5	87.9	7.2
	30	25.6 ± 1.8	85.2	7.2
Tall fescue endophyte-free seed	150	118.5 ± 6.6	79.0	5.6
	30	19.2 ± 2.4	63.9	12.6
Tall fescue endophyte-free straw	150	116.1 ± 7.2	77.4	6.2
	30	18.4 ± 1.5	61.2	7.9
Feces‡	150	130.7 ± 8.8	87.1	6.8
	30	24.4 ± 3.6	81.3	14.8

* $n = 9$.
† CV = coefficient of variation.
‡ Fecal material was collected from steers consuming a diet of endophyte-free straw.

determining metabolism and route of excretion was evaluated. A protocol for detection of LA in serum is currently under development (AM Craig, 2005, personal communication). Recovery of LA when known concentrations (high [150 ng/ml] or low [30 ng/ml]) of LA were added into each matrix (Table 1) indicated that percentage of recovery was greatest at the high level (150 ng/ml). Recovery of LA obtained from feces was greatest when samples were spiked with 150 ng/ml LA versus those spiked with the low level of LA (30 ng/ml; $P < 0.05$). Average LA recovery was 82.3% for samples spiked with a high level of LA versus 74.3% recovered for those spiked with 30 ng of LA/ml. At the low level of LA (30 ng/g), the greatest recovery ($P < 0.05$) was observed in the urine, ruminal fluid, and feces, with an average recovery of 82.1%, compared with 62.6% recovery for the remaining 2 matrices ($P < 0.05$). At the high level of LA (150 ng/g), the greatest recovery ($P < 0.05$) was also observed in the urine, ruminal fluid,

Table 2. Repeatability of evaluation of high-performance liquid chromatography (HPLC) detection of lysergic acid in ruminal fluid, urine, feces, tall fescue seed, and tall fescue straw.

Item	High-spike lysergic acid detected*†	Recovery (%)§	CV (%)¶	Low-spike lysergic acid detected*‡	Recovery (%)‖	CV (%)
Ruminal fluid						
Day 1	119.6 ± 7.8	79.8	2.8	24.0 ± 0.4	79.9	1.8
Day 2	124.5 ± 2.1	83.0	1.7	24.3 ± 0.8	80.8	3.2
Day 3	126.3 ± 5.0	84.2	4.0	24.0 ± 0.6	80.1	2.6
Urine						
Day 1	137.2 ± 10.1	91.4	7.4	26.5 ± 1.5	88.2	5.6
Day 2	129.3 ± 9.9	86.2	7.7	26.5 ± 0.6	88.2	2.9
Day 3	135.8 ± 9.7	90.5	7.2	23.2 ± 1.9	77.3	8.0
Feces						
Day 1	137.9 ± 6.4	91.9	4.7	27.1 ± 1.5	90.3	5.5
Day 2	129.4 ± 11.6	86.3	9.0	27.2 ± 1.6	90.5	5.8
Day 3	125.4 ± 1.5	83.6	1.2	21.4 ± 2.0	71.3	9.2
E− tall fescue seed						
Day 1	128.1 ± 7.1	85.5	5.5	17.7 ± 0.5	59.1	2.9
Day 2	119.1 ± 1.3	79.4	1.1	17.1 ± 1.7	62.0	9.9
Day 3	116.5 ± 2.2	77.7	1.9	21.4 ± 1.9	71.5	8.9
E− free tall fescue straw						
Day 1	111.5 ± 6.1	74.4	5.5	19.6 ± 0.8	65.4	4.1
Day 2	123.8 ± 5.1	82.5	4.1	18.3 ± 1.5	60.9	8.2
Day 3	112.8 ± 2.3	75.2	2.0	17.2 ± 1.0	57.2	5.9

* $n = 3$.
† 50 ng of lysergic acid/ml was added to each matrix.
‡ 30 ng of lysergic acid/ml was added to each matrix.
§ High-level effect of day ($P > 0.05$), recovery from feces and urine > seed, straw, and ruminal fluid ($P < 0.05$).
‖ Low-level matrix × day interaction ($P = 0.01$), recovery from feces and urine > seed, straw, and ruminal fluid ($P < 0.05$).
¶ CV = coefficient of variation.

Table 3. Limit of quantification (LOQ) and limit of detection (LOD) of the method for each matrix.

Item	Ruminal fluid	Urine	Endophyte-free seed	Endophyte-free straw	Feces
LOQ, ng/ml	5.45	18.41	24.24	14.50	36.00
LOD, ng/ml	1.64	5.52	7.26	4.35	10.80

and feces, with average recovery of 85.1% versus 78.2% average recovery for straw and seed.

To evaluate repeatability of the assay, samples were prepared in triplicate at 2 concentrations and injected on 3 separate days (Table 2). When 150 ng of LA/g was added to the 5 matrices of interest, there was no difference in recovery ($P = 0.41$) on any of the 3 days. However, recovery differed ($P < 0.05$) among matrices, with the same trends for recovery (Table 1). Additionally, CV within and between runs were calculated and all CVs were <10%. A lower concentration of LA was also evaluated (30 ng/ml). Matrix affected recovery ($P < 0.05$), and there was no day × matrix interaction ($P = 0.73$).

The LOQ and LOD for all matrices are shown in Table 3. Limit of quantitation is defined as the minimal injected amount that gives a precise measurement. Ruminal fluid and urine LOQ were 5.5 and 18.4 ng/ml, respectively. Seed, straw, and feces had higher LOQ of 24.2, 14.5, and 36.0 ng/g, respectively. Limit of detection indicates the amount of LA that can be detected but not quantified, the LOD values were below LOQ values with LOD of 1.64, 5.52, 10.80, 7.26, and 4.34 ng/ml in ruminal fluid, urine, feces, and endophyte-free seed and straw, respectively.

Samples of ruminal fluid, urine, and feces were collected from steers consuming a diet containing 400 ng of ergovaline/g. These samples, in addition to the endophyte-infected tall fescue straw, were analyzed for LA to determine the usefulness of this assay for detection of LA under physiologic conditions in diets containing E+ feedstuffs (Table 4). All matrices resulted in values above the LOD and LOQ for the assay, proving that this assay is sufficiently sensitive for use in assessment of metabolism of LA.

The stability of LA in the extraction solvent (1 : 1 pure water : acetonitrile) was tested by use of a time series sampling. Lysergic acid was not obviously degraded in the extraction solvent even after 48 hours, indicating that LA does not degrade in the extraction solvent (Table 5).

Discussion

The lowest recoveries of spiked material regardless of amount of LA added were from the E− seed and straw diets, indicating that LA values in feedstuffs may be underestimated. Recovery values for LA recovered from urine, ruminal fluid, and feces were similar to ergot alkaloid recovery values from wheat as previously reported.[8] Those authors added various ergot alkaloids to wheat, and calculated recoveries ranged from 79.1 to 95.9%. However, LA was not one of the ergot alkaloids tested. Therefore, this data set represents the first attempt to quantify LA in ruminal fluid, urine, feces, and tall fescue seed and straw using HPLC.

Greatest recovery of spiked LA was from urine, feces, and ruminal fluid, whereas lowest LA recovery was observed from spiked endophyte-free tall fescue seed and straw. This indicates that, at lower levels of detection, LA may be somewhat variable and that the LOQ of the assay is important. The amount of LA recovered from samples of animals fed the E+ diet indicates that the amount found in real samples was above the LOQ, with the exception of feces, which had a level between the LOD (10.80 ng/g) and the LOQ (36.00 ng/g).

In addition to precise analysis of LA, the procedure described here provides diagnosticians with a tool that is consistent on a day-to-day basis. The development of this assay is crucial to the understanding

Table 4. Analysis of ruminal fluid, urine, and feces collected from steers consuming endophyte-infected tall fescue straw with 400 ng of ergovaline/g.

Item	No. samples injected*	Lysergic acid (ng/g)	CV (%)†
Endophyte-infected tall fescue straw	15	24.2 ± 0.90	1.2
Ruminal fluid	22	13.3 ± 0.34	6.5
Urine	22	26.3 ± 1.58	5.2
Feces	20	20.6 ± 1.34	2.4

* Each sample was injected in duplicate.
† Duplicates with CV >10% were re-extracted.

Table 5. Stability of lysergic acid in a 1 : 1 acetonitrile : water mixture used for extraction of lysergic acid from seed, straw, and feces.

Sampling time*	Lysergic acid added (ng/ml)	Lysergic acid detected (ng/ml)†	Recovery (%)	CV (%)	Change from 0 hr (%)
0 hr	150	127.1 ± 6.8	84.7	5.4	–
6 hr	150	138.4 ± 1.2	92.3	0.86	+8.5
18 hr	150	126.7 ± 10.2	84.49	8.1	−0.2
48 hr	150	129.7 ± 5.4	86.5	4.18	+2.0

* 0-hr sample taken immediately after lysergic acid was added.
† $n = 9$.

of the metabolic fate of ergot alkaloids and how they contribute to fescue toxicosis. The ability to analyze for total, as well as individual ergot alkaloids will eventually define whether fescue toxicosis in livestock results from total ergot alkaloid load or a specific ergot alkaloid such as ergovaline or LA. Additionally, biotransformation and bioavailability of the ergot alkaloids to LA can now be addressed with the advent of a more sophisticated analysis of LA. This will lead to improvements in livestock management strategies and design of tall fescue cultivars that are more livestock friendly.

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Sources and manufacturers

a. Bar Diamond, Parma, ID.
b. Thermo Forma, Marietta, OH.
c. EMD Chemicals, Gibbstown, NJ.
d. Beckman, Fullerton CA.
e. JT Baker, Phillipsburg, NJ.
f. Fisher, Pittsburgh, PA.
g. Sigma, Dellfonte, PA.
h. Fisher, East Hills, NY.
i. Discovery DSC-SCX SPE, Bellfonte, PA.
j. Alltech, Deerfield, IL.
k. Mallinckrodt, Phillipsburg, NJ.
l. Branson Ultrasonics Corporation, Danbury, CT.
m. Nanosep NF GHP P/N, Pall Life Sciences, East Hills, NY.
n. Eppendorf, Westbury, NY.
o. National Scientific Company, Duluth, GA.
p. Perkin Elmer 250 Binary Pump, Shelton, CT.
q. Perkin Elmer Series 200 autosampler, Shelton, CT.
r. Perkin Elmer LC 240, Shelton, CT.
s. Phenomenex, Torrance, CA.
t. SAS Institute Inc., Cary, NC.

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