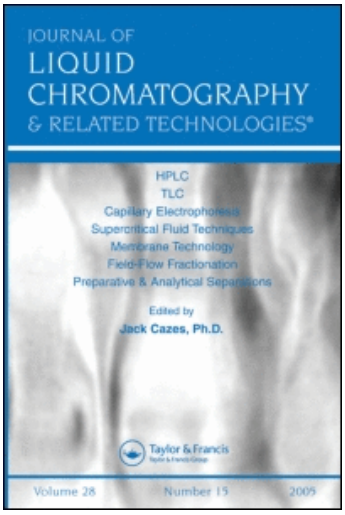

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Neely, Frank L.(1992) 'Determination of Monensin in Fermentation Broth by HPLC with Post-Column Derivatization', Journal of Liquid Chromatography & Related Technologies, 15: 9, 1513 — 1522
To link to this Article: DOI: 10.1080/10826079208018304
URL: http://dx.doi.org/10.1080/10826079208018304

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(9), 1513-1522 (1992)

DETERMINATION OF MONENSIN IN FERMENTATION BROTH BY HPLC WITH POST-COLUMN DERIVATIZATION

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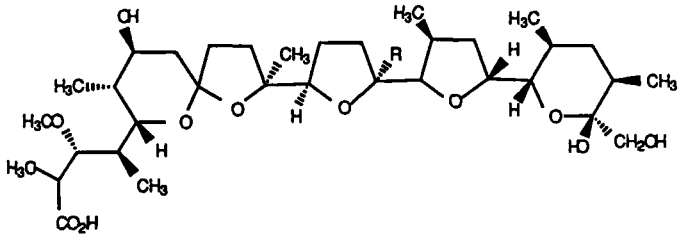
ABSTRACT

A rapid method was developed for the determination of monensin in fermentation broth. A post-column reaction was optimized to enhance the sensitivity and selectivity of the analysis. The method was shown to be reproducible and sufficiently rugged for the analysis of a complex matrix.

INTRODUCTION

Monensin is a polyether antibiotic produced by *Streptomyces cinnamonensis*[1] used primarily for growth promotion in cattle and to combat coccidiosis in poultry. Monensin A and monensin B, shown in figure 1, are the two major factors produced in fermentation broths. The focus of this work was to develop a rapid screening assay which could resolve the two monensin factors in fermentation broth. Several techniques, including microbiological[2,3], thin-layer chromatographic[4-6], and high performance liquid chromatographic assays[7-10], have been described in the literature for the determination of monensin. Microbiological assays have long been used in the screening of antibiotics, but lack the desired factor specificity for this application. The chromatographic procedures that have been described for feeds use refractive index detection or require pre-column derivatization to facilitate detection.



monensin A R= CH_2CH_3

monensin B R= CH_3

Figure 1. Molecular structure of monensin A and B.

Refractive index detection is not sufficiently selective for the analysis of fermentation broth and pre-column derivatization is not amenable to high sample throughput. Based on previous work narasin[11], also a polyether antibiotic, an HPLC assay with post-column reaction detection has been developed to enhance selectivity and therefore minimize analysis time. The post-column reaction is based on the Komarowski reaction[12], a reaction of secondary alcohols with benzaldehydes to produce a red colored product. A chromatographic method is described and characterized.

EXPERIMENTAL

Chemicals All solvents, unless otherwise described, were of HPLC grade. Monobasic ammonium phosphate and vanillin were of reagent grade. Monensin standard (95.3% monensin A, 3.9% monensin B) was obtained from Lilly Research Laboratories (Indianapolis, IN).

Instrumental The HPLC apparatus was composed of the following components: a BioRad AS100 autosampler, two Beckman 1108 HPLC pumps, an Applied Biosystems 757 detector, and a FIAtron FH40 reactor coupled to an FH50 controller. The post column reactor consisted of 10 feet of 0.010" diameter stainless steel tubing wrapped tightly around the heater core. A Regis Little Champ column with a C_{18} packing was used.

Sample Preparation Samples were prepared by adding a 45 mL portion of methanol to 5 mL of fermentation broth. The suspension was homogenized with a mechanical grinder and allowed to

settle for an hour at 5°C. If particulate was still present after this period of time, the supernatent was filtered to 0.45 μm with a syringe filter (Acrodisk, Gelman Associates). If necessary, the solution was diluted with methanol to lower the concentration of monensin within the linear range of the assay. Standards were prepared by dissolving the appropriate weight of sodium monensin in methanol to give a 1.0 mg/mL standard.

Chromatographic Conditions Mobile phase consisted of 85% methanol and 15% buffer. The buffer was prepared by dissolving 1.0 g monobasic ammonium phosphate in water and adjusting the pH of the solution to a value of 3.0 with phosphoric acid. The post column reagent solution was composed of 3% vanillin and 3% concentrated sulfuric acid in methanol. The vanillin was dissolved first and the solution chilled prior to the addition of the sulfuric acid. The addition of sulfuric acid generates considerable heat and, if added too quickly, results in significant coloration of the reagent. Once prepared, the solution is stable for at least two weeks if maintained at 0°C. The flowrate of each solutions was 1.5 mL/min. The injection volume was 10 μL and the detector wavelength set at 520 nm. The post column reactor was held at 120°C.

RESULTS AND DISCUSSION

Prior to optimizing the reaction conditions, the methanol:buffer ratio was varied over the the domain 4:1 to 19:1 to achieve baseline resolution of monensin factors A and B in minimum time. The value of log (k') varied linearly with percent methanol in the mobile phase, indicating a reversed phase chromatograph mechanism for both species. The slopes of the resultant plots were -0.062 and -0.052 for monensin A and B, respectively. The ratio of 5.67:1 of methanol:broth gave a resolution of 1.5 and was used as the mobile phase throughout the optimization.

The pH value of the mobile phase buffer was varied over the domain 2.0 to 6.0 with integer increments. Interestingly, no trend was observed of retention, peak shape, or sensitivity with pH value. The choice of pH 3.0 as the final buffer pH was essentially arbitrary, although acidic conditions are desirable for column longevity.

Optimization of the post column reaction conditions was initially conducted under flow injection analysis conditions with the sample injected directly into the reagent stream. Duke[13] has proposed that the first step of the reaction of secondary alcohols with benzaldehydes in acidic media is the dehydration of the alcohol to form a carbonium ion intermediate. Not

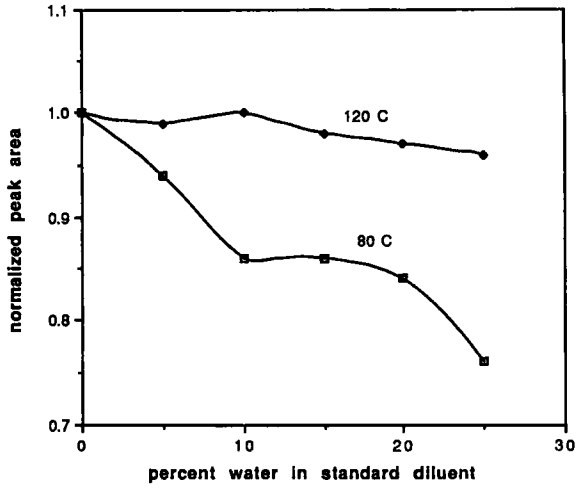


Figure 2. Effect of water in diluent on reaction of monensin with vanillin in FIA mode.

surprisingly, we found that the presence of water in the sample diluent had a pronounced effect on the magnitude of the detector response when the reaction was conducted at 80°C. As shown in figure 2 the intensity of the response decreased with increasing water concentration, indicating that water is a stronger base than vanillin. This effect was less dramatic at 120°C . In the post column derivatization mode, the water content of the solution is 92.5%, which exhibited no decrease in signal intensity at 120°C. Therefore 120°C was selected as the operating temperature of the assay.

The optimization of reagent concentration was conducted in post column HPLC mode by preparing solutions of vanillin and sulfuric acid in methanol in combinations of 0, 1 , 3, and 5%. A ·.0 mg/mL standard was analyzed in triplicate with the sixteen combinations of reagent. Table I lists the average area of the monensin A peak. No response was observed with reagent lacking vanillin or sulfuric acid. The magnitude of the response increased with vanillin and sulfuric acid concentration. Monensin B behaved similarly. However, discoloration of the reagent also increased with the same trend. The index of reagent solution performance was defined as the peak area of the standard divided by the reagent solution absorbance at 520 nm

Table I. Effect of reagent composition on detector response[a].

% H_2SO_4	% vanillin	$Area_{monensin\ A}$	$Area_{monensin\ B}$	$A^{b}_{520\ nm}$
1.0	1.0	0.03	0.03	0.021
1.0	3.0	0.10	0.09	0.029
1.0	5.0	0.15	0.15	0.049
3.0	1.0	0.16	0.16	0.041
3.0	3.0	0.43	0.42	0.088
3.0	5.0	0.59	0.52	0.196
5.0	1.0	0.31	0.30	0.222
5.0	3.0	0.68	0.66	0.438
5.0	5.0	1.00	1.00	0.680

[a] Average of three determinations. For convenience, areas have been normalized.

[b] Absorbance of reagent solution after six hours standing at room temperature. Measurement performed in 1 cm cuvette.

after six hours at room temperature. Figure 3 shows a contour plot of the resulting response surface. From this plot, the optimum reagent solution composition is 3% vanillin and 3% sulfuric acid.

Figure 4 shows a typical chromatogram of a fermentation broth extract obtained under the optimized conditions. Monensin A and B are well resolved and exhibit acceptable peak symmetry. Two small peaks of unknown origin, were observed, one at the solvent front, the other eluting after monensin A. The magnitude of detector response was directly proportional to monensin concentration over the domain 0.25 to 2.0 mg/mL. The ratio of the response of monensin A to monensin B was 0.86, suggesting that the two factors may react with vanillin at different rates.

The efficiency of extraction of monensin from the fermentation broth was evaluated by preparing suspensions of broth and methanol at several ratios. Table II lists the mass of monensin extracted as a function of methanol:broth ratio. For presentation purposes, the entries to the table have been normalized. Complete extraction (>95%) was obtained at a methanol:broth ratio of 5, although further dilution may be desirable to adjust the sample concentration within the linear range of the assay.

The effect of the sample matrix was evaluated by spiking monensin standard into a methanol extract of fermentation broth. Table III lists the recovery of monensin as a *function of percent*

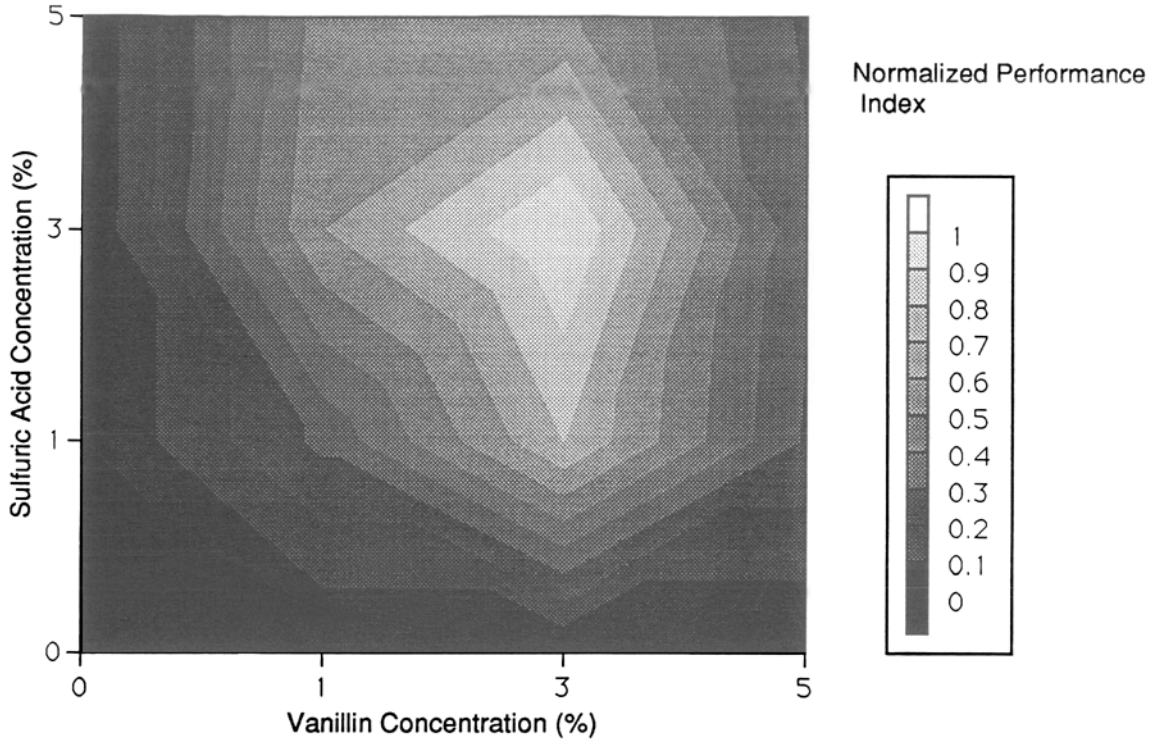


Figure 3. Contour plot of response surface of reaction of monensin with vanillin in the presence of sulfuric acid. Performance index defined as peak area of 1.0 mg/mL in post-column HPLC mode divided by absorbance of reagent solution at 520 nm after standing for six hours at room temperature. Data normalized for presentation purposes.

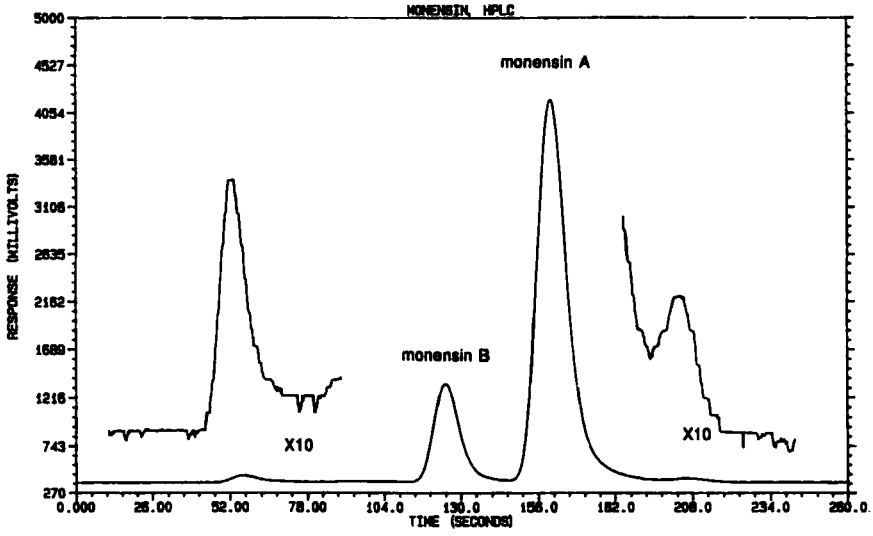


Figure 4. Chromatogram of a typical fermentation broth sample. Chromatographic conditions are described in the text.

Table II. Extraction efficiency as a function of methanol:broth ratio.

methanol:broth	mass extracted monensin[a]
4	0.81
6	0.88
8	0.93
10	1.00
12	0.99
14	1.00

[a] Average of three determinations. Results have been normalized for simplification.

Table III. Evaluation of matrix effect.

Percent of native concentration	Recovery
100% (native)	NA
117%	104%
139%	99%
216%	101%
344%	101%
average:	101%

Table IV. Recovery of spiked monensin.

Percent of native concentration	Recovery
100% (native)	NA
102%	101%
104%	99.9%
108%	100.1%
121%	99.6%
131%	97.0%
average:	99.4%

Table V. Evaluation of assay precision.

Day	Average Result[1] (mg/mL)	r.s.d.
1	0.69	1.2%
2	0.65	3.0%
3	0.68	3.0%
6	0.68	0.9%
8	0.70	1.2%
10	0.70	1.2%

[a] Average of nine injections of a single methanol extract. Result is sum of concentration of monensin A and B.

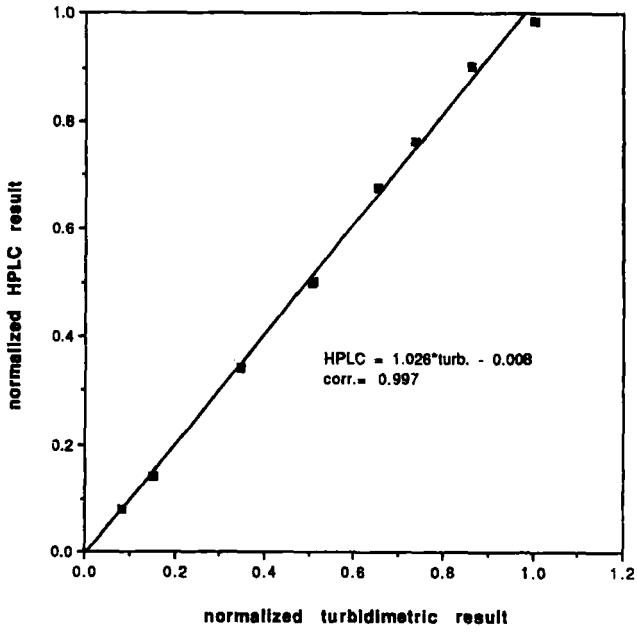


Figure 5. Comparison of HPLC data with turbidimetric data for samples obtained throughout a typical fermentation. Results have been normalized to the highest measured potency for presentation purposes.

of native monensin spiked into the sample. Over the domain 13% to 244% above native monensin concentration, the average recovery was 101%, indicating no matrix effect. Monensin was also spiked directly into the fermentation broth to evaluate extraction efficiency. The concentration regime studied in the spiking experiment was smaller than in the matrix effect study to determine if small differences in the concentration of monensin could actually be detected. The results are listed in table IV. Over the domain 2% to 31% above native monensin concentration, the average recovery was 99.4%, indicating quantitative recovery of spiked monensin.

The assay was evaluated for short and long term precision by analyzing a control sample, a methanol extract of fermentation broth, with nine replicates daily. The pertinent data is given in table V. Within a given day, the typical coefficient of variation observed was about 1.2%. The average result varied by less than 3% over 7 days, indicating stability of monensin in the sample matrix as well as reproducibility of the assay.

A series of fermentation samples, encompassing the typical concentration range found throughout a fermentation, were analyzed by HPLC and a microbiological assay[2]. The results of the assays are compared in figure 5. The HPLC result is the sum of factors A and B. Over the concentration domain studied, the results of the HPLC assay correlate well with turbidimetric results. Linear regression of the data yields a correlation coefficient of 0.997 and an essentially origin intercept. The slope of 1.026 compares well with the theoretical value of 1.000.

CONCLUSIONS

A rapid HPLC assay with post-column derivatization suitable for the analysis of large numbers of samples, has been described and characterized. The accuracy of the method has been verified by comparison to biological activity.

ACKNOWLEDGEMENTS

The author wishes to extend his appreciation to Mr. Cecil A. Perry for providing microbiological assays.

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