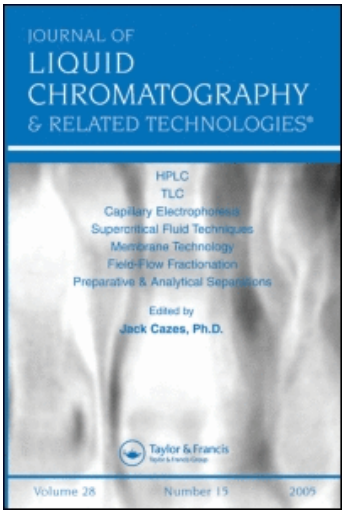

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THE ELECTROCHEMICAL DETECTION OF PENICILLINS IN MILK

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ABSTRACT

Penicillins can be detected in milk samples using pulsed amperometric detection following reversed-phase high performance liquid chromatography. A detection limit of $2*10^{-6}$ M was achieved for penicillin G, with similar values obtained for the other penicillins tested. To improve these detection limit values, an on-column dual-solvent concentration scheme was adapted. A concentrating solvent forced the deposition of penicillins onto the C-18 column. Afterward, the normal chromatographic eluent carried the penicillins off the stationary phase and allowed a separation. Work with standard solutions proved the viability of the scheme and also demonstrated the linearity of detector response at low concentrations. Using the concentration procedure, a detection limit of $2*10^{-7}$ M was found for penicillin G in milk solution, and in general the concentration scheme allowed detection limits for all of the penicillins to be improved by approximately a factor of 10.

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INTRODUCTION

The penicillins are a group of antibiotics commonly prescribed for a variety of bacterial infections. The natural products penicillin G and 6-aminopenicillanic acid are produced by fermentation of *Penicillium chrysogenum*; further members of this class are produced by synthetic modification of these natural products (1). The impact penicillins have made on human medicine is well understood; not as well known is the degree to which penicillins have been adapted for use in veterinary medicine for treatment of infections and as a prophylactic during surgical procedures (2-6).

One group of animals that may be treated with penicillins are dairy cattle (7,8). These drugs are commonly applied by dairy farmers to fight lung and udder infections in their herd. It is legal to use penicillins to treat these infections as long as the bovine system is clear of the antibiotic during milking. Nevertheless, there is the potential for the transfer of penicillins into milk. This may have serious consequences to the milk consumer, as the incidence of allergic response to penicillins may be as high as 10% (9). While this response may be just the appearance of a mild skin rash, in acute cases the response may be an anaphylactic reaction, which can be fatal. The Food and Drug Administration has recently announced plans for a nationwide milk screening program for penicillins, as well as other drugs, in response to criticism from consumer groups (10).

Analytical methodology for penicillin screening in milk must meet stringent standards. Milk is a fairly complex substance, providing a challenging sample matrix. As well, the screening tests must feature detectability to trace concentration levels. Several different analytical approaches have been used for these screening tests. Microbiological assays have shown the requisite sensitivity, but they are time-consuming, lack specificity, and do not lend themselves well to

quantitative work (11). Colorimetric assays lack both sensitivity and specificity. Recent work has focused on high performance liquid chromatography (HPLC) usage to provide selectivity in conjunction with a sensitive detector. Several groups have detected penicillins by direct UV absorbance following HPLC (12-15) with detection limits reported as low as 0.1 ppm. The low wavelengths chosen (typically 200-230 nm) led to poor detector selectivity, which was problematic when applied to milk samples. Despite selectivity problems, detection limits to .03 ppm for milk samples were reported (11) for a method incorporating a chemical concentration step into the sample preparation procedure before injection into the HPLC. Moats (16, 17) was able to detect less than 10 ppb of penicillin, but only after extensive sample preparation that involved a concentration step and "heart cutting" (18), where a fraction from a preparative HPLC trial is collected and rechromatographed on the analytical HPLC system.

Other detector options are available for coupling with HPLC. Several indirect methods and derivatization schemes have allowed more selective photometric detection at higher wavelengths (19-22) but have lacked sufficient sensitivity. Fluorescent penicillin adducts can be produced using o-phthaldialdehyde (23,24), but this is limited to species having a primary amine group on their side chain. Electrochemical detection has been reported using amperometric oxidation after photolytic derivatization with a UV flashlamp (25). Recent work in this group (26,27) has shown that pulsed amperometric detection (PAD) at a gold electrode can provide a simple approach to detecting all penicillins without the necessity of derivatization. PAD allows sensitive response to the penicillins either by direct oxidation of the compound or indirectly by the suppression of residual current from background processes, depending on the waveform chosen. Detector sensitivity is comparable to direct UV detection, but

the selectivity of PAD is superior considering the UV wavelengths necessary. This work sought to apply this detection methodology following reversed-phase HPLC to milk samples containing penicillins.

EXPERIMENTAL

Materials and Reagents

Penicillins were obtained from Sigma Chemical Co. (St. Louis, MO, USA). A listing of the penicillins used and their abbreviations are given in Table 1. Reagent grade acetic acid from Fisher (Pittsburgh, PA, USA) and sodium acetate from Baker (Philipsburg, NJ, USA) were used to produce acetate buffer solutions. For milk extractions, GC/pesticide grade hexane and HPLC grade methylene chloride from Alltech (Deerfield, IL, USA) were used. HPLC grade acetonitrile and methanol from Fisher were used as mobile phase organic modifiers. Water for aqueous solutions was distilled and deionized before use. Chromatographic mobile phases were vacuum filtered through an Alltech 0.2-micrometer nylon

TABLE 1
A Listing of the Penicillins Used

PENICILLIN	ABBREVIATION
Amoxicillin	Amox
Ampicillin	Amp
Cloxacillin	Cloxa
Dicloxacillin	Dicloxa
Methicillin	Meth
Nafcillin	Naf
Oxacillin	Oxa
Penicillin G	Pen G
Penicillin V	Pen V

filter and sonicated before use. A nylon 0.45-micrometer syringe-tip filter was used to screen samples before their injection into the HPLC.

Chromatographic Apparatus

A Waters (Milford, MA, USA) 625 Gradient LC System was used as the primary pump for all HPLC work. A flow rate of 2.0 ml/minute was standard for most applications. The pump was run in "silk mode", a Waters feature designed to reduce pump noise. The injection loop had a volume of 50 microliters. When the dual pump concentration scheme was adapted, a Spectra Physics (San Jose, CA, USA) Isochrom LC pump was used as the concentrating pump. To apply the concentrating system, the main valve in the Waters 625 was replumbed as can be seen in Figure 1. In the concentrating mode, the Spectra Physics pump concentrated milk samples onto the column and then pumped to waste. Normally, the flow rate for the Spectra Physics pump was 3 ml/minute and the concentrating solvent was .02 M acetate buffer. In this mode, the Waters 625 LC system pumped the normal mobile phase, the eluting solvent, through the detector and then out to waste. When the valve was switched to the analytical mode, the concentrating pump was diverted directly to waste. The analytical pump now delivered the mobile phase to the column eluting the concentrated milk sample. Finally, the concentrated sample entered the detector for analysis. The standard gradient program used for penicillin separation, adapted from reference 27, is given in Table 2.

A Waters 8 x 10 Radial-Pak Compression Module housed a 10-micrometer μBondaPak C-18 Radial-Pak cartridge stationary phase. The cartridge had an internal diameter of 8 mm and a length of 100 mm. All separations were done at ambient laboratory temperature (ca. 20 +/- 2 degrees C.) Sample preconcentration was always done on the analytical column.

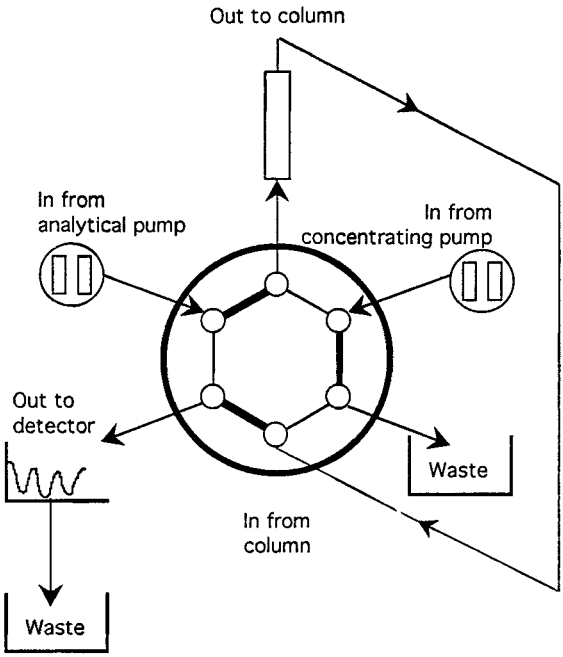

Figure 1. Valve setup in concentration mode.

TABLE 2
Gradient Program For Penicillin Separations
(Flow Rate 2 ml/min)

TIME (MIN)	COMPOSITION
Initial	15% Acetonitrile 10% Methanol 75% .02 M Acetate Buffer
0-15	Linear Ramp
15	30% Acetonitrile 0% Methanol 70% .02 M Acetate Buffer
15 +	Isocratic Hold

Electrochemical detection used the Waters 464 Pulsed Electrochemical Detector. A thin-layer cell was utilized that had an electrode block containing dual gold electrodes in the series configuration. The upstream element was used as the working electrode, while the downstream electrode served as the counter electrode for this work. To minimize detector noise, the detector was configured in the floating ground mode throughout this project, which served to ground the detector at a virtual point rather than to the chassis of the instrument. A Ag/AgCl reference electrode was used in the thin-layer cell. An indirect PAD waveform, given below, was used for all milk applications.

E1 = 1300 mV for .166 seconds

E2 = 1500 mV for .166 seconds

E3 = -200 mV for .333 seconds

Chromatographic data was collected with a Gateway (N. Sioux City, SD, USA) 386SX computer using a Keithley MetraByte (Taunton, MA, USA) Chrom-1AT interface board. Recorded PAD data for quantitative studies was treated with a fourier transform smoothing algorithm to minimize high frequency noise.

Milk Sample Preparation

Skim milk of various brands was purchased at local grocery stores. Milk samples were prepared by spiking measured volumes of the skim milk with known amounts of penicillins. Proteins were then precipitated with two volumes of acetonitrile. The solution was left standing for five minutes during which time the precipitated proteins settled to the bottom. The aqueous portion was decanted and filtered by suction. Fats were then extracted with an equal volume of 1:1 methylene chloride:hexane. The aqueous phase was centrifuged at 3000 rpm for 10 minutes. Finally, the remaining solution was diluted 3:1 with 0.02 M acetate buffer and passed through a 0.2 micrometer nylon filter. For detection without on-

column preconcentration of the milk samples, there was no dilution with acetate buffer.

RESULTS AND DISCUSSION

Direct injection of milk samples was not advisable to insure a reasonable HPLC column lifetime, yet an important concern was to make the sample preparation as simple and quick as possible. The complexity of the milk sample matrix forced past workers to introduce various lengthy sample preparation schemes, as noted in the Introduction. It was hoped that the selectivity of PAD would permit the investment of less sample preparation time. A detailed description of the basic sample preparation procedures can be found in the Experimental section. This procedure removed largely the proteins and fats, leaving behind lower molecular weight water-soluble species. However, it was known that this portion was still relatively complex in composition (28).

Injections of the milk extract spiked with penicillins illustrated that most of the response to the milk sample matrix was confined to a large void response eluting within 4 minutes of injection. The major effect on the penicillin separation was that any species eluting before ampicillin would coelute with this void response. Although ampicillin suffers from a slight overlap with this region, it can be clearly discerned as well as all following peaks. (see Figure 2). A set of experiments were devised to find the percentage of penicillins recovered from the milk samples, and to examine the reproducibility of response. Five injections of a standard solution (typically $1*10^{-4}$ M) in a water matrix were made into the C-18 column. Three separate milk extractions were run, and a series of 5 injections made from each of the extracts. The resulting statistics from these experiments, based on peak height measurements, are summarized in Table 3. The recovery

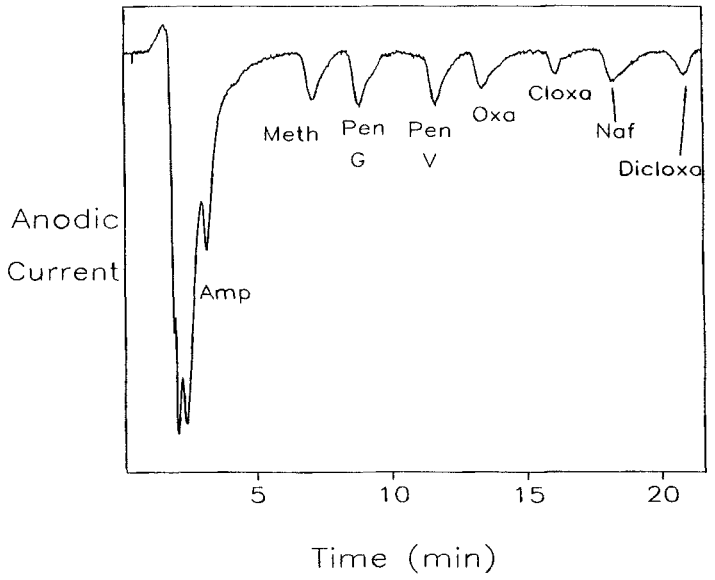

Figure 2. Direct detection of 8 pencillins within a milk sample. 50 µl injections on a µBondapak C-18 column. All penicillins are at $1*10^{-4}$ M. Waveform and gradient given in the Experimental section.

TABLE 3
Statistics from HPLC separations in Milk.

	Amp	Meth	Pen G	Pen V	Oxa	Cloxa	Naf	Dicloxa
Gradient k' value	0.82	3.57	4.61	6.42	7.55	9.59	10.83	12.74
Recovery % from milk samples	56.4	91.3	87.7	84.9	78.1	66.3	63.1	71.0
Standard solution RSD (%)	4.19	2.24	2.38	2.13	3.32	2.84	8.57	2.74
Intra-extract variability (RSD, %)	4.93	5.12	4.55	4.96	8.57	9.73	8.51	6.73
Inter-extract variability (RSD, %)	3.50	2.68	8.90	3.35	6.55	9.50	10.4	4.14

percentages are good considering the difficulty of recovering the penicillins quantitatively during the extraction step. It is interesting to note the inverse correlation between k' and recovery percentage. This is likely due to the more non-polar penicillins not being extracted efficiently into the aqueous phase during sample preparation. Ampicillin is an exception to this trend, which may be due in part to its overlap with the void response. For the most part, the variabilities are below 10% relative standard deviation. The later eluting peaks tended to have larger variabilities, but this was largely due to the fact that these are expressed in terms of relative standard deviations (RSD). The later peaks are also the smallest ones, inflating the RSD's relative to the earlier, larger peaks. If the variabilities are expressed in terms of absolute standard deviation, these later eluting compounds are only slightly less reproducible than their earlier counterparts.

Detection limits for direct injection of the milk extracts containing penicillin spikes are given in the first row of Table 4. These values are from 2-5 times higher than what can be obtained with a cleaner sample matrix. As the recovery percentages in Table 3 attest, this is mainly due to an increased noise level when

TABLE 4
Milk Detection Limits

	Amp	Meth	Pen G	Pen V	Oxa	Cloxa	Naf	Dicloxa
Detection limit for direct 50 µl injections of milk samples (µM)	2	2	2	2	3	4	4	7
Detection limit for on-column concentration of milk samples (µM)	0.3	0.3	0.2	0.2	0.2	0.3	0.2	0.3

using the milk samples rather than a loss in sensitivity. The detectability of the later-eluting peaks suffered somewhat due to the expected chromatographic peak broadening, and some slight degree of improvement could be seen by employing alternative HPLC allowing them to elute earlier (but causing coelution of the initial peaks). Otherwise the PAD response was close to the maximum that could be obtained with our detector.

Other routes to improve detectability by employing a concentration step were examined. The first attempt at concentration was directed toward simply evaporating the sample under reduced pressure to lower the volume and concentrate the penicillins. The problem with this approach, though, was that the concentrated solutions were simply too viscous for HPLC analysis. Those samples that were injected caused clogging problems. Further steps to thin the sample were examined, but there appeared to be no way to avoid a time-consuming and labor-intensive process. Thus, an alternative method for concentration was sought.

Another method tested to improve detectability was an on-column concentration scheme. The basic plumbing for the system is shown in Figure 1 and described in the Experimental section. Early chromatographic work during this project tested aqueous mobile phases containing only buffer and/or electrolyte but no organic modifier. Under these conditions injected penicillins were infinitely retained on the C-18 stationary phase and not recovered from the column. Upon decreasing the polarity of the mobile phase by the addition of ca. 30% by volume methanol and/or acetonitrile, reasonable retention times were achieved. Therefore, an aqueous solvent was used to concentrate the penicillins on the C-18 column, followed by a switch to the regular gradient program containing organic modifier to elute the concentrated sample. By doing this, penicillin solutions that would be

undetectable using the direct method could be concentrated to the point of detectability. One drawback to this type of procedure was that a much larger sample volume would be required. This was much less a concern for bovine milk samples than for samples from human body fluids. One major advantage to this type of concentration was that it could easily be automated to run as an on-line process. A similar method could probably be developed using an off-column concentration step with a solid phase extraction cartridge.

Before any milk samples were analyzed, extensive testing of the method on standard penicillin solutions was undertaken. Figure 3 shows the detection of 10 ppb penicillin V dissolved in .02 M acetate buffer. The sample solution was concentrated for 20 minutes using a flow rate of 3 ml/minute before switching to an elution solvent of 30% acetonitrile/70% .02 M acetate buffer. This solvent was used for samples containing only a single penicillin instead of the standard gradient program in order to save time during repetitive trials. The large baseline shift at around 2.5 minutes is due to the transition from the concentrating solvent to the eluting solvent at the detector. The concentration scheme kept the elution solvent flowing through the thin-layer detector cell even during the concentration period to minimize detector noise. However, upon switching from concentration to elution a fairly large volume of the aqueous concentrating solution would be trapped in the system and had to pass through the detector cell, causing the perturbation at short times following valve switching.

As long as the final eluted concentration of the penicillin was less than $3.5*10^{-4}$ M, linear detector response could be expected for the concentration method (27). At greater concentrations the current was less than predicted by extrapolation of the linear region, as current was proportional to adsorbed analyte surface coverage rather than concentration, giving isotherm-like response (26). A

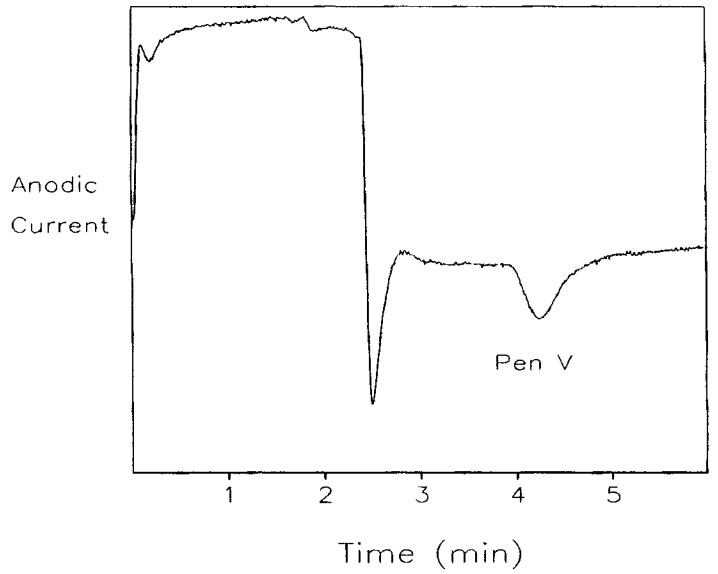

Figure 3. 10 ppb penicillin V detected after concentration. 20 minutes of concentration at 3 ml/min. Analytical solvent was 30% acetonitrile/ 70% .02 M acetate buffer, 2 ml/min. Waveform given in the Experimental section.

plot of peak area vs. concentration was made for a series of penicillin V solutions, each concentrated for 6 minutes at 3 ml/min. The linearity was good; the statistics below describe the plot for solutions ranging from 15 to 35 ppb in concentration.

$$y = -28.360 + 5.9880x \quad R^2 = 1.000$$

Alternatively, response was also linear with concentration time. A 20 ppb penicillin V solution was concentrated for various times ranging from 4 to 10 minutes at a fixed flow rate. A plot of peak area vs. concentration time gave a linear plot as described by the following statistics.

$$y = -17.450 + 15.450x \quad R^2 = 0.998$$

One major limitation of other methods designed to achieve trace penicillin detection is that they are limited to measuring a single penicillin specie. This

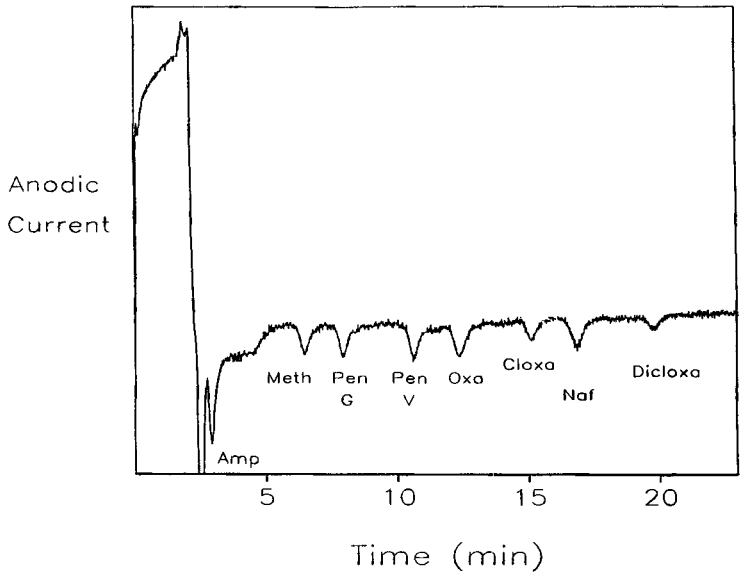

Figure 4. Separation of 8 penicillins at $2.6*10^{-8}$ M after concentration. 30 minutes of concentration at 3 ml/min. μBondapak C-18 column. Waveform and gradient given in the Experimental section.

concentration method can be used for samples containing multiple penicillins. Figure 4 shows a sample containing $2.6*10^{-8}$ M each of 8 different penicillins after on-line concentration.

When applied to milk samples, usage of the on-line concentration method allowed detection limits to be lowered versus determination by direct injection. As noted in the Experimental section, the milk extract had to be diluted before use with the concentration system. Without this thinning step, the viscous solution could not be pumped through the system without column clogging and HPLC pump pressure overloads. Figure 5 shows a separation of 8 penicillins in milk, all at $1*10^{-5}$ M. The milk was concentrated on-column for 8 minutes at a flow rate of 3 ml/min before the separation took place. Although longer times were useful

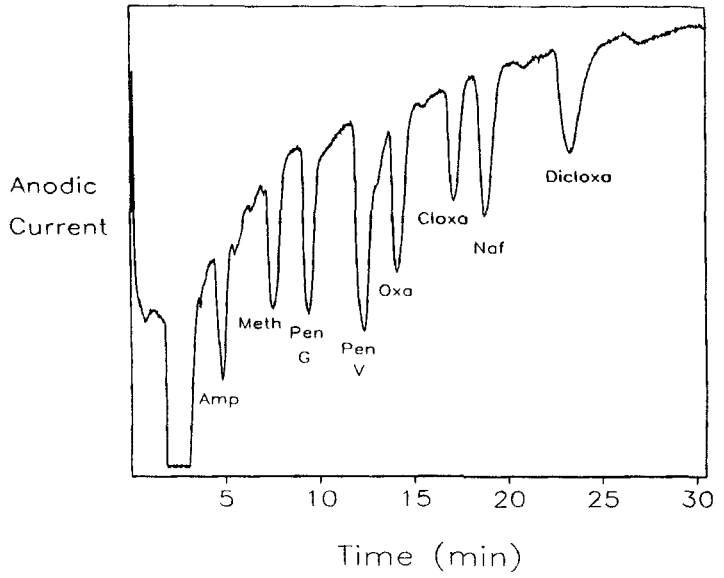

Figure 5. The separation of 8 penicillins in a milk sample following concentration. 8 minutes of concentration at 3 ml/min. All penicillins were present at $1*10^{-5}$ M in the milk. μBondapak C-18 column. Waveform and gradient given in the Experimental section.

when concentrating standard solutions, better output was obtained for the milk samples with concentration times of less than 10 minutes (at 3 ml/min), as the concentration step for the penicillins served to accentuate a few species from the milk sample matrix that were not previously found to be problematic during direct injections of the milk extract. The interference from these species ultimately limited detectability; a detection limit of $2*10^{-7}$ M was achieved for penicillin G, and in general detection limits were improved by ca. a factor of 10 for all the penicillins, as shown in Table 4. All of these values were obtained with 8 minutes of concentration time, requiring 24 ml of milk sample.

CONCLUSION

Detection of penicillins within milk samples can be done directly to low micromolar concentrations using PAD following HPLC. Application of the on-column concentration scheme allowed 8 penicillins to be separated and quantitated to less than micromolar concentrations. Although this scheme requires the use of 2 HPLC pumps, the concentration scheme is relatively quick, can be completely automated if necessary, and would not need any further labor than that already required for preparation of milk samples. In contrast to some current procedures, this method is adaptable for sensitive detection of any one of 8 penicillins, either as solitary analytes or mixed together.

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REFERENCES

1. G. J. Tortora, B. R. Funke and C. L. Case, Microbiology: An Introduction,3rd Ed., Benjamin/Cummings, Redwood City, CA, 1989.

2. P. A. Okewole, E. M. Uche, I. L. Oyetunde, P. S. Odeyemi and P. B. Dawal, Lab. Anim., 23, 275, (1989).

3. E. P. Tulleners, J. Am. Vet. Med. Assoc., 198, 1765, (1991).

4. T. L. Seahorn and J. Schumacher, J. Am. Vet. Med. Assoc., 199, 368, (1991).

5. S. Y. Gardner, V. B. Reef and P. A. Spencer, J. Am. Vet. Med. Assoc., 199, 370 (1991).

6. K. A. Johnson and S. C. Roe, J. Am. Vet. Med. Assoc., 192, 1573, (1988).

7. J. M. Reimer, R. W. Sweeney and J. Saik, J. Am. Vet. Med. Assoc., 192, 1297, (1988).

8. R. R. Leder, V. M. Lane and D. P. Barrett, J. Am. Vet. Med. Assoc., 192, 1299 (1988).

9. A. G. Gilman, L. S. Goodman and A. Gilman, The Pharmacological Basis of Therapeutics, 6th Ed., Macmillan, New York, 1980.

10. K. Schneider, Chicago Tribune, Dec. 28, 1990, Section 1, Page 5.

11. H. Terada and Y. Sakabe, J. Chromatogr., 348, 379, (1985).

12. F. Jehl, P. Birckel and H. Monteil, J. Chromatogr., 413, 109, (1987).

13. W. A. Moats, J. Chromatogr., 317, 311, (1984).

14. A. M. Lipczynski, Analyst, 112, 411, (1987).

15. A. Marzo, N. Monti, M. Ripamonti, E. A. Martelli and M. Picari, J. Chromatogr., 507, 235, (1990).

16. W. A. Moats, J. Chromatogr., 507, 177, (1990).

17. W. A. Moats, J. Assoc. Off. Anal. Chem., 75, 257, (1992).

18. J. Carlqvist and D. Westerlund, J. Chromatogr., 344, 285, (1965).

19. A. Besada and N. Tadros, Mikrochimica Acta, 2, 225, (1987).

20. E. Mendez-Alvarez, R. Soto-Otero, G. Sierra-Paredes, E. Aguilar-Veiga, J. Galan-Valiente and G. Sierra-Varcuno, Biomed. Chromatogr., 5, 78, (1991).

21. W. T. Kok, J. J. Havax, W. H. Voogt, U. A. T. Brinkman and R. W. Frei, Anal. Chem., 57, 2580, (1985).

22. B. Morelli and M. Mariani, Anal. Letters, 20, 1429, (1987).

23. M. E. Rogers, M. W. Adlard, G. Saunders and G. Holt, J. Chromatogr., 297, 385, (1984).

24. M. E. Rogers, M. W. Adlard, G. Saunders and G. Holt, J. Chromatogr., 257, 91, (1983).

25. C. M. Selavka, I. S. Krull and K. Bratin, J. Pharm. Biomed. Anal., 4, 83, (1986).

26. L. Koprowski, E. Kirchmann and L. E. Welch, Electroanalysis, 5, 473, (1993).

27. E. Kirchmann and L. E. Welch, J. Chromatogr., 633, 111, (1993).

28. J. M. Orten and O. W. Neuhaus, Human Biochemistry, 10th ed., C. V. Mosby Co., St. Louis, MO, 1982.

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