A Comparison of Some Chromatographic Detectors for the Analysis of Volatile *N*-Nitrosamines

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The analysis of volatile *N*-nitrosamines by thermal energy analysis (TEA) and gas chromatography (GC) is compared with GC–Coulson electrolytic conductivity detector (GC–CECD), GC–high-resolution mass spectrometry (GC–MS), and thin-layer chromatography (TLC). Quantitative chromatograms without interfering peaks were obtained by GC–TEA on crude aqueous distillates and on final methylene chloride extracts following extensive cleanup. For GC–CECD, GC–MS, and TLC data of equal quality were only obtained on the final methylene chloride extract after the extract had been concentrated by a factor of 50. Because of the high sensitivity of the GC–TEA, even for dilute extracts, a chromatographic peak corresponding to trace levels of *N*-nitrosopiperidine was found in several samples.

Because they are extremely potent carcinogens, there is currently much interest (Bogovski and Walker, 1974) in developing selective and sensitive analytical methods for the analysis of volatile nitrosamines in cured foodstuffs such as bacon, fish meal, pepper cures, etc. Although it is generally recognized (Telling et al., 1971) that gas chromatography–mass spectrometry is the most reliable and proven method for unequivocal confirmation of the identity of nitrosamines isolated from foods, there is a need for simpler techniques that can be used for routine screening purposes.

Palframan et al. (1973) made a comparison of the performance of the gas chromatograph–Coulson electrolytic conductivity detector (GC–CECD) and the gas chromatograph–alkali flame ionization detector (GC–AFID) with the gas chromatograph–mass spectrometer (GC–MS) for the analysis of volatile nitrosamines from foodstuffs. Only slight differences in the sensitivity between the GC–CECD and GC–AFID were found.

The thermal energy analyzer (TEA), which is claimed to be both highly selective and highly sensitive to *N*-nitrosamines (Fine et al., 1974, 1975), has recently been interfaced directly to a gas chromatograph (Fine and Rounbehler, 1975). This paper compares three different chromatographic techniques, viz. GC–CECD, GC–MS, and thin-layer chromatography, with the GC–TEA technique for the trace analysis of volatile *N*-nitrosamines in foodstuffs.

EXPERIMENTAL SECTION

The equipment used was operated under the preferred conditions normally prevailing in the two laboratories.

Apparatus. *Coulson Electrolytic Conductivity Detector (CECD).* The CECD, as described by Coulson (1965), is a reasonably specific and sensitive detector of compounds containing nitrogen and, by varying the operating conditions, also of compounds containing sulfur and halogens. It has been widely used as the detector of choice in the gas chromatographic estimation of volatile nitrosamines (Bogovski and Walker, 1974). The CECD used here was interfaced to a Varian Aerograph (Model 2700) gas chromatograph and operated in the pyrolytic mode (Sen et al., 1974). The column was a 6 ft × $^1/_8$ in. stainless steel tube packed with 25% Carbowax 20m and 2% NaOH on 60–80 mesh Chromosorb P. After injection, the column effluent was vented. After venting the column was temperature programmed at the rate of 10 °C/min from 120 to 170 °C. The Coulson furnace was maintained at 360–380 °C, with the cell voltage at 30.

Chart I. Summary of Extraction and Cleanup Procedures

Sample 25–50-g sample
↓vacuum distillation
160-ml aqueous distillate (step 1)
| K_2CO_3
| extracted twice with methylene chloride
| acid wash of organic layer
| back-wash of organic layer with K_2CO_3 solution
↓dried over Na_2SO_4, filtered
Total volume 500 ml (step 2)
| concentrated
| transferred into heptane
| alumina column cleanup
↓eluted with methylene chloride (50 ml)
Total volume 50 ml (step 3)
↓concentrated to 1 ml
Total volume 1.0-ml final concentrate (step 4)

High-Resolution Mass Spectrometer (MS). A Varian Mat (Model 311A) high-resolution mass spectrometer equipped with an electron impact ionization source was used, interfaced to a Varian Aerograph (Model 2700) gas chromatograph by means of a Watson Biemann separator. The mass spectrometer was operated in the specific ion monitoring mode for NO^+ (m/e 29.9980) at a resolution of 5000 (Sen et al., 1976). The mass spectrometer operating conditions were: source temperature, 250 °C; emission current, 3 mA; electron voltage, 70 eV; accelerating voltage, 3 kV. The same GC column as used for the CECD detector was used. The column was kept at 140 °C for 4 min and then programmed at the rate of 10 °C/min to 180 °C and then maintained at 180 °C.

Thermal Energy Analyzer (TEA). A Thermo Electron (Model 502) TEA was used, interfaced to a specially designed column gas chromatograph (Fine and Rounbehler, 1975). The TEA operating conditions were: furnace temperature, 300 °C; cold trap temperature, −150 °C; reaction chamber pressure, 5 mmHg. The GC column was a 6 ft × $^1/_8$ in. i.d. stainless steel tube packed with 15% FFAP (15 g of FFAP on 100 g of Chromosorb W, acid washed, DCMS treated, 80–100 mesh). The nitrogen carrier gas flow rate was 30 ml/min. The column was temperature programmed from 140 to 220 °C at a rate of 5 °C/min.

Thin-Layer Chromatography (TLC) of N-Nitrosopyrrolidine. For the final detection, the heated TLC plate

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Table I. Comparison of Results

	N-Nitroso compd	GC-TEA, μg/kg[a] 1	2	3	GC-Coulson, μg/kg, 4[a]	GC-MS, μg/kg, 4[a]	TLC
Fish meal	NDMA	105	94	98	92	90	
Cooked bacon fat	NDMA	Trace		4.5	8.8	6.2	
	NPIP	Trace		Trace			
	NPYRR	31		25		25	40
Cooked lean bacon	NDMA					0.2	
	NPYRR					7	8

[a] Steps 1, 2, 3, and 4 of the Sen analysis procedure.

was observed inside a Chromatoview chamber (Ultra-Violet Products, Inc., San Gabriel, Calif.), with both the short-wave and long-wave transilluminator ultraviolet lamps being used (Sen et al., 1973).

Procedures. Extraction, cleanup, and concentration of all food samples were carried out in one of the two collaborating laboratories (NPS). Aliquots were withdrawn from the analytical procedure at key points (see Chart I), and sealed in preweighed glass ampules. At the completion of the analysis by GC–MS and/or GC–CECD and/or TLC, the remnants of the final concentrates, if any, were also sealed into preweighed glass ampules. The ampules were delivered to the second laboratory (DHF) for analysis by GC–TEA. Both laboratories used the same calibration standard mix of four *N*-nitrosamines, *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodibutylamine (NDBA), and *N*-nitrosopyrrolidine (NPYRR), supplied by the International Agency for Research on Cancer. Analytical standards were introduced immediately before and immediately after the introduction of the food extracts. Peak height was used as the basis for quantitation throughout.

The extraction and cleanup procedures which were used were similar to those described by Sen et al. (1974). In the first step a portion (25–50 g) of the sample was vacuum distilled from alkaline solution (see Chart I). Approximately 1 ml of the 160-ml aqueous distillate was removed for direct analysis by GC–TEA. In the second stage the distillate was made alkaline and extracted with methylene chloride and the methylene chloride extract was acid washed, back washed with alkali, and then dried over sodium sulfate. The extract was filtered and made up to 500 ml. Of the 500 ml of combined methylene chloride extract 5 ml was removed for analysis on the GC–TEA. The remaining methylene chloride extract was then concentrated, transferred into heptane, and purified by chromatography on a basic alumina column. The column was eluted with 50 ml of methylene chloride. Of the 50 ml of eluate 1 ml was removed for GC–TEA analysis. In the final stage, the 49 ml from the third stage was further concentrated on a Kuderna Danish evaporator to 1.0 ml. Recoveries from spiked foodstuffs were typically 80–90% for NDMA and 60–80% for NPYRR. For the GC–CECD, GC–MS, and TLC techniques, only the final 1.0 ml of concentrate was used.

RESULTS

A fish meal sample was prepared according to the extraction and cleanup procedures described above. Figure 1a is the GC–TEA chromatogram for 20 μl of the aqueous distillate from the first vacuum distillation stage, prior to all cleanup. Although the baseline was not stable, the apparent NDMA peak is clearly discernible. The amount of NDMA found is 105 μg/kg, based on the original sample. Figure 1b shows the GC–TEA chromatogram after dilution with methylene chloride from 160 to 500 ml. The NDMA peak, showing 94 μg/kg NDMA based on the original sample, is still visible, with a signal-to-noise ratio (peak to peak) of 3:1. Figure 1c shows the GC–TEA chromatogram of the third stage concentrate, before final concentration to 1.0 ml. The amount of NDMA found was 98 μg/kg. In all three GC–TEA chromatograms, no extraneous peaks with a signal-to-noise ratio of greater than 2:1 were observed. Figure 2a is the GC–CECD for 4.9 μl of the final 1.0 ml of concentrate from the fourth stage. The amount of NDMA found was 92 μg/kg, based on the original sample. It should be noted that three peaks with a signal-to-noise ratio of 3:1 and one peak with a signal-to-noise ratio of 6:1 are clearly visible. Figure 2b is the GC–MS chromatogram of 2.2 μl of the final 1.0 ml of concentrate. The amount of NDMA present was found to be 90 μg/ml, with no peaks having a signal-to-noise ratio greater than 3:1 being visible. The analytical results are

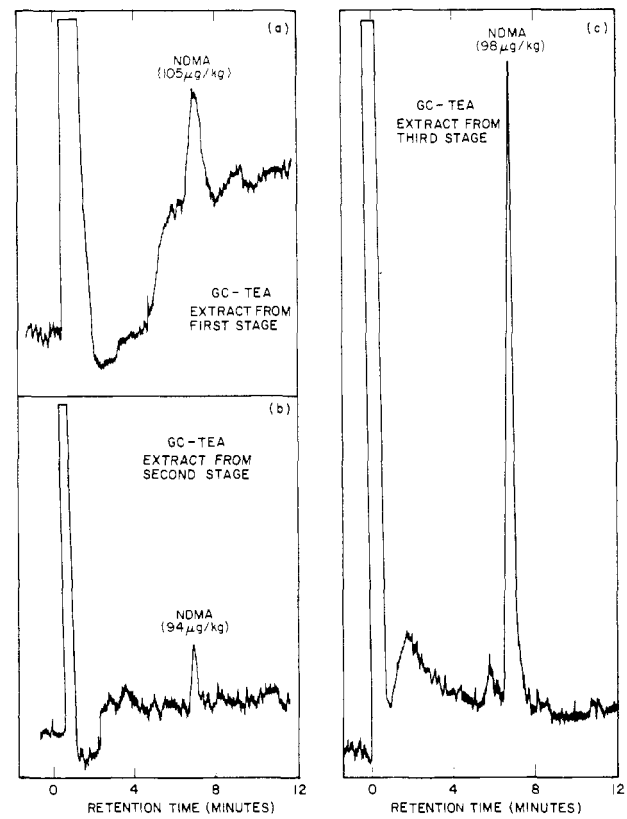

Figure 1. Chromatograms from the cleanup of a fish meal sample. Figure 1a is the GC-TEA chromatogram of 20 μl of the aqueous distillate from the first stage immediately after vacuum distillation (25 g of fish meal diluted to 160 ml of extract). Range on TEA × 4. Figure 1b is the GC-TEA chromatogram of 20 μl of the methylene chloride extract from the second stage (25 g of fish meal diluted to 500 ml of CH_2Cl_2). Range on TEA × 2. Figure 1c is the GC-TEA chromatogram of 20 μl of the methylene chloride extract from the third stage, before concentration (25 g of fish meal diluted to 50 ml of CH_2Cl_2). Range on TEA × 2.

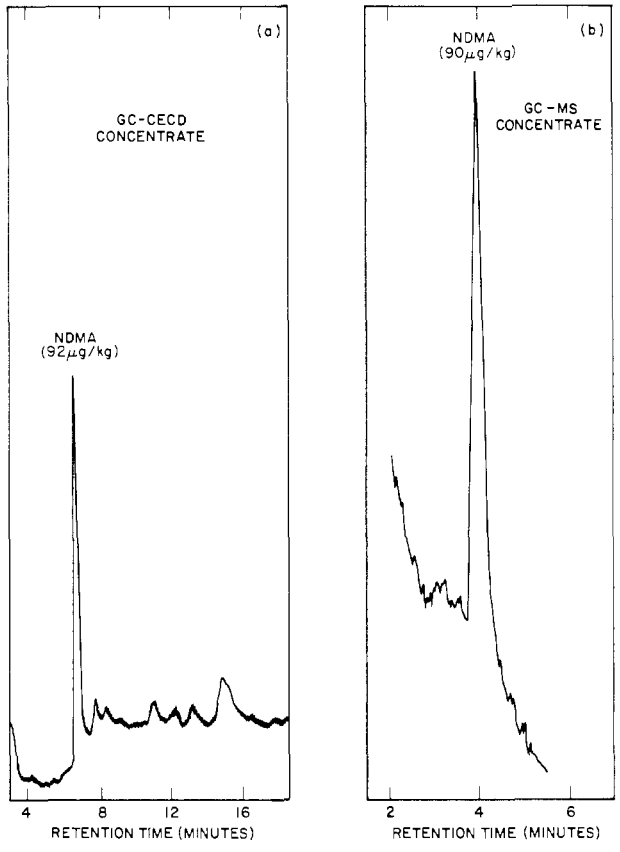

Figure 2. Chromatograms from the cleanup of a fish meal sample. Figure 2a is the GC-Coulson chromatogram of 4.9 μl of the final methylene chloride concentrate (25 g of fish meal concentrated to 1 ml of CH_2Cl_2). Range on Coulson × 1. Figure 2b is the GC-MS chromatogram of 2.2 μl of the final methylene chloride concentrate (25 g of fish meal concentrated to 1 ml of CH_2Cl_2). Range on MS × 0.03.

summarized in Table I. The larger amount of NDMA found on the GC–TEA after the first and third stages (the signal-to-noise ratio in the second stage was not sufficiently large to give a precise result) as compared with that found after the final stage by GC–CECD and GC–MS is consistent with the known recovery efficiency (approximately 90%) of the analytical method.

In Figure 3 a comparison is made of the chromatograms of extracts taken from a cooked bacon fat sample. Figure 3a is the GC–TEA chromatogram of 20 μl of the aqueous distillate from the first stage. The apparent NDMA peak is not visible above the noise, but traces corresponding in retention time to nitrosopiperidine (NPIP) and 31 μg/kg *N*-nitrosopyrrolidine (NPYRR) are clearly visible. Figure 3b is the GC–TEA chromatogram from the third stage methylene chloride concentrate. NDMA is found at the 25 μg/kg level. Again, both GC–TEA chromatograms show no extraneous peaks. Figures 3c and 3d are the GC-CECD and the GC–MS chromatograms, respectively, of the 1.0-ml final methylene chloride concentrate. The GC–CECD shows several extraneous peaks, one of which is greater than the NDMA and NPYRR being searched for. The GC–MS chromatogram is uncluttered, the only two peaks observed being those due to NDMA and NPYRR. In the case of the cooked bacon fat, the observed concentration levels are considerably smaller than for the fish meal analysis. The GC–TEA results compare reasonably well with the GC-MS, viz. 4.5 μg/kg for TEA compared with 6.2 μg/kg for MS, but the 8.8 μg/kg observed for the CECD is probably too large; the difference may be due to

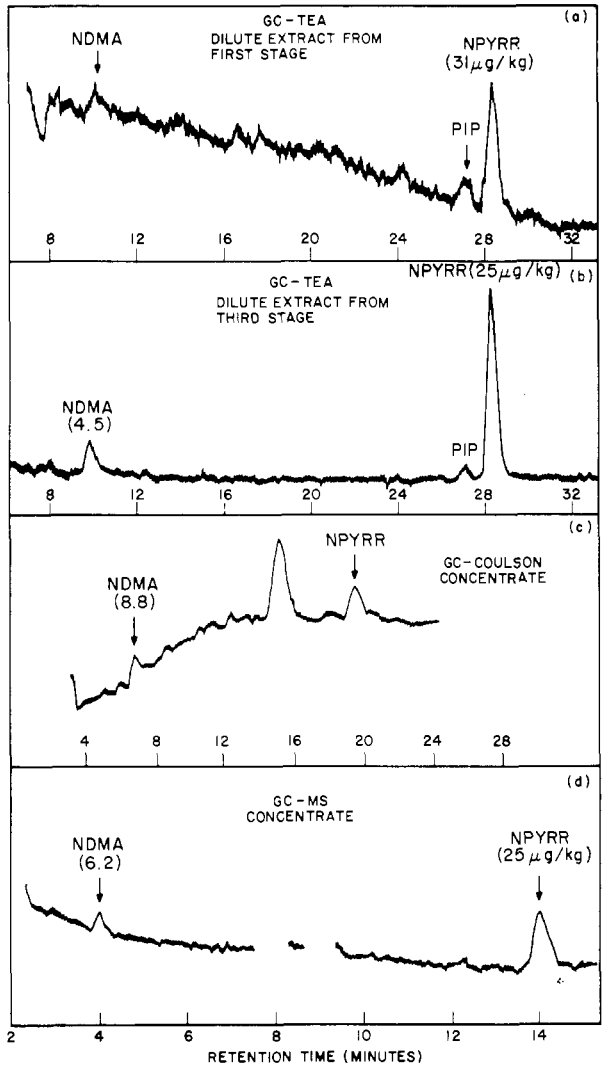

Figure 3. Chromatograms from cleanup of a cooked bacon fat sample. Figure 3a is the GC-TEA chromatogram of 20 μl of the aqueous distillate from the first stage, immediately after vacuum distillation. Range on TEA × 2. Figure 3b is the GC-TEA chromatogram of 20 μl of methylene chloride extract from the third stage, before concentration. Range on TEA × 4. Figure 3c is the GC-Coulson chromatogram of 4.1 μl of the final methylene chloride concentrate. Figure 3d is the GC-MS chromatogram of 2.3 μl of the final methylene chloride concentrate. Range on MS × 0.01.

interfering co-eluting compounds or a lack of linear response at extremely low levels in the pyrolytic mode. Although a peak corresponding in retention time to NPIP was found by GC–TEA, it was not observed by GC–MS. It is not clear whether the GC–MS would have had the sensitivity to detect the NPIP. It should be stressed that for all the figures shown here the GC–TEA chromatograms were made with solutions which were at least 50 times more dilute than the chromatograms shown for the GC–CECD and the GC–MS.

Figure 4 is a comparison of the chromatogram taken from a cooked lean bacon sample. The GC–TEA chromatograms from the first and third stages show no evidence for either NDMA or NPYRR. The GC–CECD shows a trace of NDMA and a trace of NPYRR. The NPYRR concentration was estimated to be about 8 μg/kg in the original sample by TLC. The GC–MS chromatogram likewise shows a trace for NDMA and also some (7 μg/kg) of NPYRR. The 20-μl extracts from the first and third stages were too dilute to show up on the GC–TEA.

Table II. Comparison of TLC and GC-TEA for NPYRR

Sample[a]	GC-TEA, μg				TLC, μg of NPYRR
	NDMA	NDEA	NPIP	NPYRR	
Cayenne pepper cure	2.7	0.1	0.3	1.2	0.8
Rat feed, cooked bacon fat	0.23			0.9	0.9
Cooked bacon fat	0.3		0.08	1.9	1.8

[a] Not analyzed by Coulson detector or by MS.

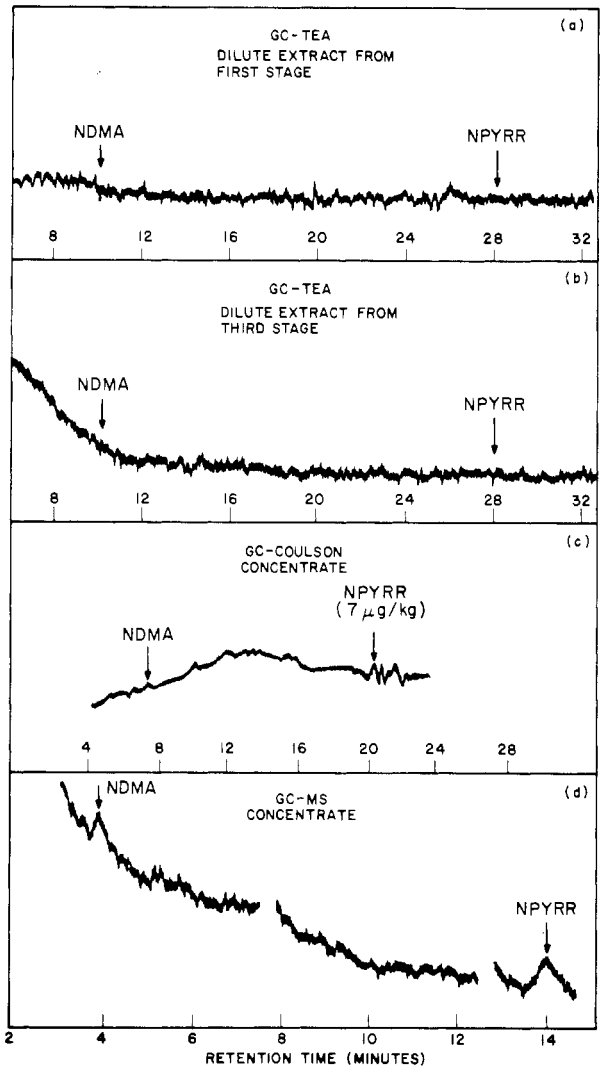

Figure 4. Chromatograms from cleanup of a lean cooked bacon sample. Figure 4a is the GC-TEA chromatogram of 20 μl of the aqueous distillate from the first stage, immediately after vacuum distillation. Range on TEA × 2. Figure 4b is the GC-TEA chromatogram of 20 μl of methylene chloride extract from the third stage, before concentration. Range on TEA × 4. Figure 4c is the GC-Coulson chromatogram of 4.1 μl of the final methylene chloride concentrate. Range on Coulson × 1. Figure 4d is the GC-MS chromatogram of 2.3 μl of the final methylene chloride concentrate. Range on MS × 0.01.

The presence of NPYRR found by GC–MS was confirmed by TLC (see Table I). Similarly, a positive TLC response for NPYRR was found for the cooked bacon fat sample.

The final 1.0-ml concentrate from the final cleanup stage was compared on GC–TEA and TLC for extracts from a cayenne pepper cure, a rat feed containing cooked bacon, and a cooked bacon fat sample. The results, reported as micrograms in the final concentrate, are shown in Table II. The results are virtually identical for the rat feed and the cooked bacon sample, with the results for the cayenne pepper cure being slightly greater by GC–TEA than TLC.

In addition, small amounts of other *N*-nitrosamines such as NDMN, diethylnitrosamine, and NPIP were also apparently found by GC–TEA.

DISCUSSION

The identification of the various *N*-nitrosamines by GC–TEA was by coincidence of retention time between standard *N*-nitrosamines and peaks occurring in the chromatograms from the food samples. The basis for this article was the comparison of the quantitative height of the peaks occurring at the same retention time as that of the standard *N*-nitrosamines. Peaks with a signal-to-noise ratio of greater than 3:1 and corresponding to compounds other than NDMA and NPYRR were not observed on the GC–TEA.

Given that the data are for the micrograms per kilogram amount of *N*-nitrosamines in foodstuffs, the scatter of the data shown here is remarkably small. All four techniques give essentially similar results. The chromatograms shown in Figures 1a and 3a on the GC–TEA were made on the aqueous distillate, prior to cleanup and concentration. The chromatograms shown in Figures 1c and 3b were on the methylene chloride extract prior to concentration by a factor of 50. Because comparable results were obtained by GC–TEA on dilute and dirty extracts as compared with GC–CECD and GC–MS obtained on final clean concentrates, it would seem that the selectivity and sensitivity claims of the TEA may be justified. The GC–TEA and the GC–MS results are in excellent agreement.

The agreement between the different instrumental techniques, each based on widely differing physical and chemical principles, is remarkable, especially when it is considered that the data are for microgram per kilogram concentration levels. This is particularly true for the comparison of TLC with the GC–TEA results. The TLC data for NPYRR compare very closely with the GC–TEA results.

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Laser Light Scattering Bioassay for Veterinary Drug Residues in Food Producing Animals. 1. Dose-Response Results for Milk, Serum, Urine, and Bile

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A rapid, inexpensive, and extremely sensitive bioassay has been developed for the quantitative estimation of veterinary drug residues in food producing animals. The bioassay is based on the measurement of changes in differential light scattering (DLS) patterns from suspensions of drug-susceptible bacteria illuminated by a vertically polarized He–Ne laser. Various dose–response curves are presented for milk, bovine serum, urine, and bile specimens fortified with four representative drugs: penicillin-G, chlorotetracycline, furaltadone, and neomycin. The 2 to 3 h assay protocol yields log–linear dose–response curves for the four drugs over approximate ranges of 0.003–1, 0.03–10, 3–100, and 0.1–3 μg/ml, respectively.

The recent review article by Oehme (1973) has reemphasized the existence of possible health hazards in our food supply resulting from the use (Huber, 1971) of veterinary antibiotic drugs. Such drugs are used for two principal reasons: (1) the stimulation of growth and (2) the treatment of disease.

The Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) have been given the additional tasks of detecting and identifying residual drugs in consumable products and quantitating the amounts present. The FDA is currently examining the possibility of circumventing costly tissue assays by establishing correlations between tissue levels and those in the associated serum and urine.

During the past few years, our laboratory has been developing a completely new bioassay technique using differential light scattering (DLS) methods. The scattering data provided by such measurements depend upon the average cell size, shape, and structure as well as the size distribution and concentration of the bacterial assay culture being examined (Wyatt, 1968, 1969, 1972, 1973, 1975; Wyatt et al., 1972, 1976; Berkman and Wyatt, 1970; Berkman et al., 1970; Mellett and Wyatt, 1975; Mellett et al., 1976). Such methods have been shown to be particularly effective for detecting very low levels of antibiotics and evaluating bacterial antibiotic susceptibility in brief periods of time. Recently, these methods have been extended to the detection and quantitation of antineoplastic drugs in serum, urine, and bile (Wyatt et al., 1976; Mellett and Wyatt, 1975; Mellett et al., 1976). The success of these latter studies has been due in large part to the development of the Differential III, an automated instrument originally designed for the rapid quantitative determination of antibiotic susceptibilities (Wyatt, 1975). (Differential is a registered trademark of Science Spectrum, Incorporated.) New protocols have been developed that are directly applicable for the detection and quantitative determination of antibiotic residues in food producing animals. They are fast (quantitative results are produced in 2 to 3 h), require very small specimens (0.1 ml), and detect quite low levels of most drugs (e.g., 3 ng/ml for penicillin).

This assay technique has been confirmed (Wyatt et al., 1976; Mellett and Wyatt, 1975; Mellett et al., 1976) through extensive experience with antineoplastic drugs using both fortified specimens and specimens taken from animals undergoing drug therapy. The animal results to date have been in excellent agreement with conventional disc assay procedures (cf. Hunt and Pittillo, 1968) and radiometric determinations (Henderson et al., 1965; Bischoff et al., 1971). Similar methodologies have recently been applied successfully to the assay of human serum specimens containing antibiotics (MacLowry, 1975).

The work presented in this paper is concerned exclusively with the generation of standard dose–response curves for milk, serum, urine, and bile specimens using four representative veterinary drugs (penicillin-G, chlortetracycline, neomycin, and furaltadone). Later papers will specifically address liver, muscle, and kidney tissues, problems associated with drug binding, as well as different types of drugs.

DIFFERENTIAL LIGHT SCATTERING BIOASSAY

Figure 1 illustrates the light scattering measurement that forms the basis for the differential light scattering (DLS) bioassay. A suspension of exponential phase bacteria ($\sim 10^6$/ml) contained in a conical cuvette is placed in a fine laser beam which passes diametrically through the cuvette. A collimated detector rotates about the cuvette and generates a signal proportional to the intensity of the scattered light detected. The variation of scattered light intensity as a function of angle (detector position) is called the differential light scattering pattern (Wyatt, 1973). These patterns are recorded and processed by a small computer.

Figure 2 shows typical DLS patterns obtained from suspensions of penicillin-sensitive *Staphylococcus aureus* cells illuminated with a vertically polarized He–Ne laser (wavelength 632.8 nm). Four similar samples were prepared using suitably fortified milk whey. The control and penicillin-fortified specimens were incubated for 2 h while one sample was refrigerated to preserve the initial conditions. A normally growing culture interacting with whey containing no penicillin is the control. Dotted curves show

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