of the methyl ester analogue of hydroprene. Aside from the difference in compounds, our experiments differed from their's in length of exposure of the insects, 2 h vs. 24 h.

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Laser Light Scattering Bioassay for Veterinary Drug Residues in Food Producing Animals. 2. Preparation Procedures and Dose-Response Studies of Drugs in Bovine Tissues

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The differential light scattering bioassay technique has been extended to bovine tissues. The method is rapid and very sensitive. Tissue sample preparation has been reduced to a simple and rapid procedure in which the tissues are compressed in a gravity press and the exuded juice used directly after filtering. Selected dose–response data are presented for penicillin, chlortetracycline, streptomycin, and sulfaquinoxaline.

Bioassay methods utilizing differential light scattering (DLS) techniques have been shown to be useful for the detection and quantitation of low levels of veterinary drug residues in fluids from food producing animals (Wyatt et al., 1976b).

The present study is intended to extend these methods to tissue assays. As traditionally performed, such tissue assays are time consuming and extremely expensive (Microbiology Laboratory Guidebook, U.S. Department of Agriculture, 1976). They require extensive preparation, grinding, and extraction processes, followed by conventional agar well diffusion tests using various strains of selectivity sensitive bacteria. The Food and Drug Administration (FDA) hopes that it may eventually be possible to establish correlations between tissue drug levels and those in the associated urine and serum. This agency is currently pursuing such correlation studies along several avenues of research and our work here forms part of their program.

Prior to any formal application of our DLS techniques to the aforementioned correlation studies, it will be necessary to establish a rapid, consistent, and reliable tissue assay procedure. Accordingly, this paper presents details of a simplified tissue preparation procedure, some dose–response results for fortified tissue extracts, data on specificity in response to drug mixtures and the mathematical formalism required for quantitative interpretations.

DIFFERENTIAL LIGHT SCATTERING BIOASSAY PROCEDURES

Details of the DLS bioassay measurement procedures for the detection of drug residues are given in an earlier paper (Wyatt et al., 1976b) as well as in related articles (Wyatt et al., 1976a; Wyatt, 1973). In summary, the method consists of the following steps: (a) For each test specimen, a liquid aliquot is combined with an aliquot of an exponential phase broth culture of a highly sensitive bacterial strain and pronase; (b) a similar control liquid specimen is prepared from a drug-free source; (c) a blank consisting of the test specimen liquid combined with a bacterial-free broth and a similar blank using the control liquid specimen are prepared; (d) the mixtures are incubated for 2–3 h; (e) the mixtures are diluted (usually with isotonic saline) and allowed to equilibrate for about 30 min; and (f) the DLS patterns of the control, test, and blank specimens are recorded, compared, and a "score" calculated.

The DLS measurement itself is made following the schematic of Figure 1: The diluted specimen is placed in a cuvette which is illuminated by a fine laser beam (vertically polarized $\lambda = 632.8$ nm); a collimated detector rotates about the cuvette measuring the scattered light intensity as a function of angle; the detector signal is converted into a graphical or digital representation; changes or differences in the DLS test pattern with respect to the DLS control pattern are examined by means of a mathematical algorithm (Wyatt et al., 1976a,b) to generate a score. Most of the measurements of the ongoing program have been made using a Differential III system (Wyatt, 1975; Stull, 1973) and an algorithm designed initially for antibiotic susceptibility testing.

Tissue extract specimens are prepared by compressing a frozen 20–30-g sample for several minutes in a 5 cm diameter gravity press activated by a 10 kg mass. The resultant juice (~1 to 5 mL) is then filtered by vacuum through a Whatman No. 40 filter. Muscle juice is further processed by combining nine parts juice to one part of a 30% citrate solution. (The citrate solution is made by mixing 13.2 g of citric acid, 7.06 g of NaOH, 97 g of sodium citrate, and distilled water to make 366.7 mL. If necessary,

Science Spectrum, Inc., Santa Barbara, California 93105 (P.J.W., D.T.P., M.G.S., M.R.K.), and The Food and Drug Administration, Beltsville, Maryland 20705 (E.H.A.).

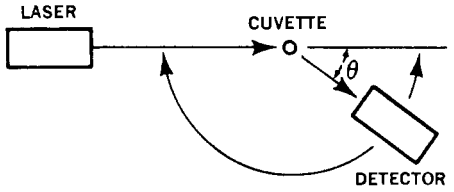

Figure 1. Differential light scattering (DLS) measurements record the intensity of scattered light as a function of the angle, θ, between the incident beam and the scattered light. DLS measurements provide information related to the number, size, size distribution, and morphology of bacteria.

pH is adjusted to 6.8 to 7.0). One part filtered tissue juice is then combined with one part pronase (2 mg/mL) and four parts bacterial broth culture prior to incubation. The background scattering level of each sample was determined using blanks prepared without bacteria. The total incubated volume is about 0.6 mL, with the initial bacterial concentration set at about 2×10^8/mL. All tissues for the present study were of bovine origin.

The proteolytic enzyme pronase has been found to be helpful in improving the optical clarity of some samples. Although the citrate solution inhibits the bacterial growth and thus slows the test, it helps to preserve the clarity of the samples and greatly enhances sensitivity for tetracycline and penicillin, presumably by chelating metal ions such as calcium.

Most drug detection assays were performed using two species exhibiting broad anitbiotic sensitivities: *Staphylococcus aureus* SS41 and *Klebsiella pneumoniae* SS886. The *Klebsiella* species is particularly sensitive to streptomycin (0.3 μg/mL), whereas the *S. aureus* is sensitive to most other drugs. Table I illustrates the useful dose–response assay ranges for various drugs using *S. aureus* SS41 and similar gram-positive strains. A variety of bacterial strains was studied for quantitative assays.

EXAMPLE OF A DOSE-RESPONSE CALIBRATION: CHLORTETRACYCLINE FORTIFIED MUSCLE JUICE

The quantitative assay of a tissue specimen requires both the identification of the drug present and the quantitation of the amount present. Although this present paper is concerned primarily with the application of DLS methodologies to the simpler detection problem (i.e., the initial screening for positive tissues), some quantitative results of an ongoing program are presented here. For the case of a single drug known to be present, the quantitation of the actual level is straightforward and follows the earlier methodologies (Wyatt et al., 1976b). This situation occurs quite frequently when, for example, an entire herd has been subjected to a specific chemotherapy for experimental or therapeutic purposes and the U.S. Department of Agriculture (USDA) inspectors have been informed a priori. As an example of such single drug assays, we shall consider the case of chlortetracycline-fortified bovine muscle.

In our earlier paper (Wyatt et al., 1976b), the basis of the Differential III score was described in detail. The score was defined by:

$$S = D_t + 3M_t \quad (1)$$

where D_t represents the average logarithmic displacement and M_t is a measure of shape differences of the two DLS patterns being compared. The shape changes of such DLS curves arise because of morphological changes in the affected bacterial cells, whereas the displacement term is a measure of relative growth inhibition.

Figure 2 shows a typical pair of dose–response scores for chlortetracycline-fortified muscle juice measured against

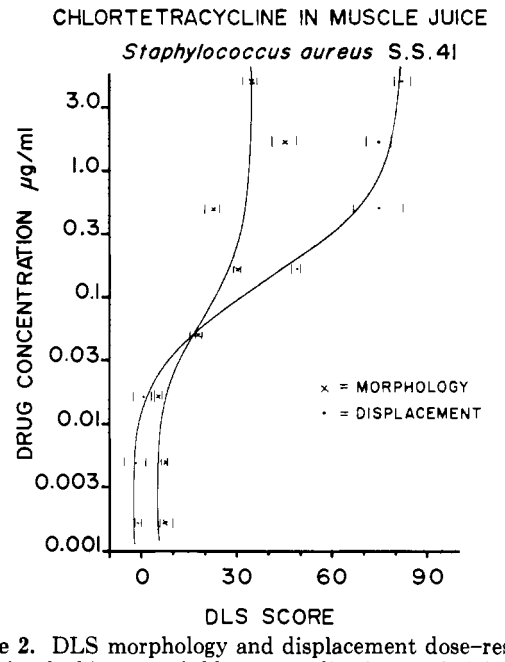

Figure 2. DLS morphology and displacement dose–response curves for the bioassay of chlortetracycline in muscle juice using *Staphylococcus aureus* 41. The DLS displacement parameter is primarily due to the difference in bacterial number between the test sample and a normally growing control. The DLS morphology parameter measures drug-induced changes in shape, size distribution, refractive index, etc.

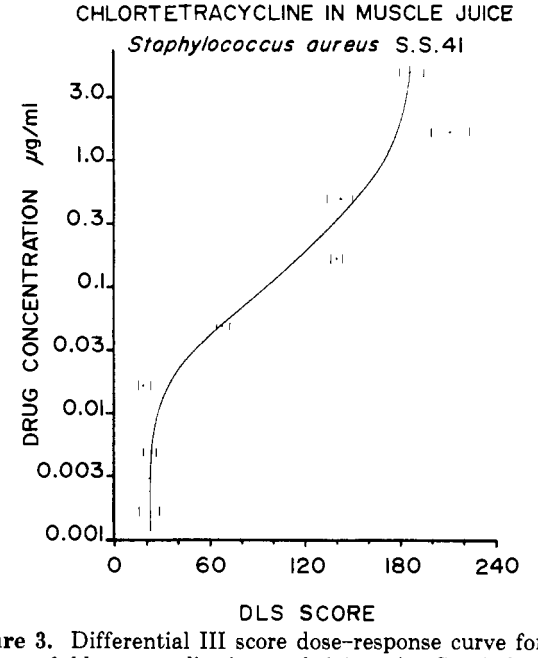

Figure 3. Differential III score dose–response curve for the bioassay of chlortetracycline in muscle juice using *Staphylococcus aureus* SS41. The Differential III score combines the DLS displacement and morphology parameters in a single measure of response, thus detecting both bacteriostatic agents and also drugs like penicillin which produce abnormal bacterial growth.

a control extract. Note that for this drug the displacement term D_t undergoes a greater net change with increasing drug presence than does the morphology index, M_t. Multiplying the latter by the factor of 3 shown in eq 1, however, yields approximately equal contributions to the total score, S. Figure 3 presents this composite score as a function of drug concentration. During the 2-h incubation period (i.e., the time during which the exponential phase bacteria *S. aureus* SS41 and the specimen juice interacted), the growth term D_t had increased by about

Table I. Assay Range: Drug Concentration (μg/mL)

Drug	Safe limit	Theraputic dose	Sample[a] BHI broth	Bovine urine	Bovine serum	Bovine whey	Bovine bile	Bovine kidney	Bovine liver	Bovine muscle
Penicillin-G	0.05	10–100	0.003–1	0.003–0.03 (6538P)	0.003–1	0.003–0.1	0.006–0.1	0.1–1.0	0.1–1.0	0.03–3.0
	0.0 milk			0.002–0.03						
Chlortetracycline	0.1	10–100	0.006–0.3	0.02–0.3 (6538P)	0.03–1	0.03–0.3		0.05–0.5	0.1–10	0.03–3.0
				0.02–0.3						
Neomycin[b]	0.1	10–100	0.6–10 (206)	0.3–3 (206)	1.5–10 (206)	(206) 0.3–10	(206) 0.06–1	(206) 3–30	(206) 3–100	100–
	1. kidney		0.1–1	0.1–3	0.1–1					
Furaltadone[c] (carcinogen)	0.02 (0)	100+	3–100	2–30 (6538P) 2–30	6–100	3–100				20–
Erythromycin			0.1–0.4							
Streptomycin			5–							
Dihydrostreptomycin			3–							
Oleandomycin			0.2–1.5							
Sulfaquinoxaline			3–100							
(potentiated with trimethoprim)		100+	(6538P) 2–100							

[a] Unless otherwise noted the assay organism is *Staphylococcus aureus* SS41. Other assay organisms are indicated by: (206) for *S. epidermidis* SS206 and (6538P) for *S. aureus* ATCC 6538P. [b] Safe limit in veal 0.25 μg/mg, in milk 0.15 μg/mL. [c] No traces allowed (F. Paglesi, FDA private communication).

Table II. Bioassay of Samples from Chlortetracycline-Treated Animals

Animal number	Chlortetracycline level, μg/mL: Urine	Serum	I.S.[b] muscle press juice (measured at 100:1 dilution)
2025	na[a]	na	1 or less
1935	40	1.0	3.5
2212	15	15. or more	Over 10
1965	70	0.2	Over 10
2043	7	0.6	Over 10
1980	30	1.0	na

[a] na = not assayed. [b] I.S. = injection site.

80 (i.e., the control relative to the initial inoculum). The relative morphology change, M_t, was about 140. Since

$$D_t = 300 \log (N_c/N_o) \quad (2)$$

the ratio (or number) of control cell concentration, N_c, to initial cell concentration, N_o, was therefore approximately 1.8.

Using the dose–response results of Figures 2 and 3 together with similar curves from our earlier paper (Wyatt et al., 1976b), it was possible to estimate the chlortetracycline levels present in specimens collected by FDA from six animals. These results are presented in Table II. Though no independent quantitative assay data were available for these specimens, the animals were known to be undergoing heavy chlortetracycline therapy. Note the high urine levels of the drug. These preliminary measurements tend to support the concept of reasonable correlation between drug levels in urine and tissue for animals undergoing continuous drug therapy. These measurements also suggest that no overwhelming chlortetracycline binding occurs in muscle samples. Of course, further binding studies are required to calibrate the assay technique.

STUDIES OF THE DLS ASSAY OF DRUG MIXTURES

Though bioassays are not generally sufficient in themselves for the final identification of an unknown substance, a "presumptive identification" can often shorten the analytical procedure required for chemical identification of residues. For this reason, studies of DLS assays of drug mixtures have been undertaken since such a procedure exactly parallels conventional methodology. Two approaches have been explored and are under current study for the problem of identifying and quantitating unknown antibiotics. First is a DLS application of the classical bioassay approach of using selective assay organisms, and second, the analysis of DLS data from a single species.

A. Selective Assay Organisms. The presumptive assay of drug *mixtures* presents a special problem that is quite different from a negative screening test designed to detect specimens containing any drug residues. For a negative screening test, a broadly sensitive organism such as *S. aureus* 41 is required that will respond to low levels of most drugs. The ideal test organism for quantitative assay, on the other hand, should be *resistant* to almost all drugs and sensitive to only one drug.

"Sensitive" and "resistant" are not absolute terms. A somewhat more precise description is given by the minimum inhibitory concentration (MIC) which stops growth in an overnight broth culture. The MIC levels of the assay organism determine the relative dynamic ranges of drug levels that can be detected in mixtures. Within a 10:1

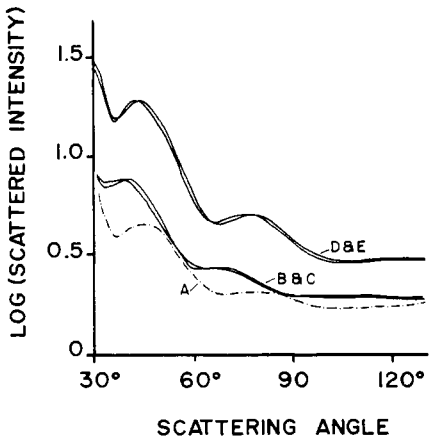

Figure 4. DLS bioassay of chlortetracycline in the presence of penicillin, streptomycin, and sulfaquinoxaline in BHI. Penicillinase is used to enhance resistance to penicillin. The test organism, *Staphylococcus* aureus SS2179, is almost completely unaffected by a mixture of penicillin, streptomycin, and sulfaquinoxaline (D) as the DLS curve falls directly on the drug-free control (E); chlortetracycline produces the same response when used alone (B) or with the other drugs (C). The initial bacterial culture before incubation, preserved by refrigeration, is shown also (A). Sulfaquinoxaline was used at 5 μg/mL and the other drugs at 1 μg/mL.

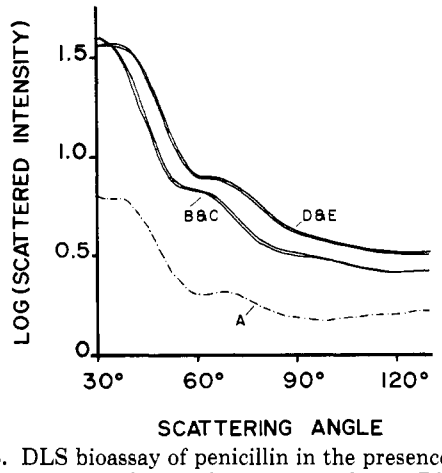

Figure 5. DLS bioassay of penicillin in the presence of streptomycin, chlortetracycline, and sulfaquinoxaline in BHI. $MgCl_2$ is used to enhance resistance to chlortetracycline. The test organism, *Proteus mirabilis* SS2531, is almost completely unaffected by a mixture of streptomycin, chlortetracycline, and sulfaquinoxaline (D) as the DLS curve falls directly on the drug-free control (E); penicillin produces almost the same response when used alone (B) or with the other three drugs (C). The initial bacterial culture before incubation, preserved by refrigeration, is shown also (A). Sulfaquinoxaline was used at 5 μg/mL and the other drugs at 1 μg/mL.

range though, examples of extreme sensitivity or resistance are not infrequent. Further development of assay strains should result in improved discrimination. At present, a mixture of two drugs of equal concentration can easily be analyzed, but in certain cases when the minor drug level is, say, only 10% of the major drug level, both drugs may have a comparable effect on the same test organism.

The ultimate ability of the technique to isolate a particular drug depends upon the availability of suitable assay organisms, though in some cases chemical reagents can be used to enhance the resistance of the assay organisms. The addition of penicillinase (β-lactamase) is an effective means of avoiding unwanted response to penicillin. The addition of *p*-aminobenzoic acid (PABA) would neutralize the sulfonamides were they present at detectable levels. Several authors (Sompolinsky and Samra, 1972) suggest that magnesium salts may block the transport of tetracycline into the cell and thus provide resistance. DLS studies have shown a tenfold increase in the level of tetracycline tolerated by a resistant organism when the assay is carried out in 0.02 M $MgCl_2$.

To demonstrate the feasibility of rapid drug identification using DLS techniques, we have determined a set of organisms suitable to assay mixtures of penicillin, chlortetracycline, streptomycin, and sulfaquinoxaline without ambiguity at levels of about 1 μg/mL: *Staphylococcus aureus* SS2179 provides tetracycline sensitivity with good resistance to the other drugs. Penicillinase improves its penicillin resistance. *Proteus mirabalis* SS2531 provides penicillin sensitivity with good resistance to the other drugs. $MgCl_2$ improves tetracycline resistance. *Staphylococcus aureus* SS40 provides moderate sulfaquinoxaline response when potentiated by trimethoprim (TMP) and exhibits excellent resistance to the other drugs. *Klebsiella* SS886 provides excellent streptomycin sensitivity, though it also responds to chlortetracycline. The search for a streptomycin sensitive assay organism which shows resistance to tetracycline has not yet been successful. Some genetic (plasmid) recombination studies have been initiated in this regard.

Figure 4 shows the detection by DLS pattern comparison of chlortetracycline in the presence of penicillin,

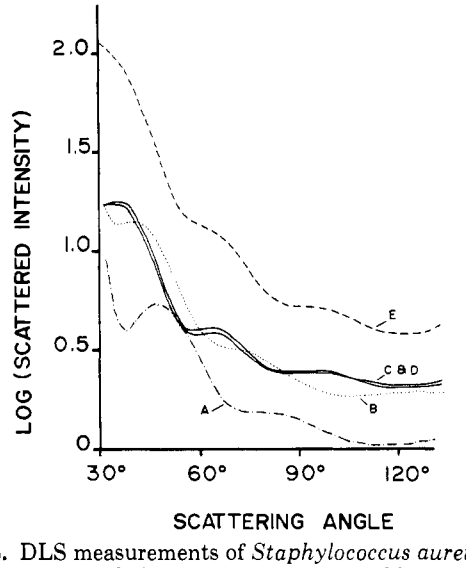

Figure 6. DLS measurements of *Staphylococcus aureus* SS41 show characteristic differences in response to chlortetracycline (C) and to streptomycin (B). When both drugs are present (D), the DLS response closely resembles that of chlortetracycline alone. DLS curves for a normally growing control (E) and a formalin killed initial inoculation (A) are also shown.

streptomycin, and sulfaquinoxaline using *S. aureus* SS2179. Figure 5 shows a penicillin assay using *Proteus mirabilis* SS2531 for the same drug mixture. The curves labeled A correspond to DLS patterns from the initial (refrigerated) inocula. Using a set of selective test organisms exhibiting the excellent discrimination shown in Figures 4 and 5, one could conceptually identify and quantitate the various drugs present by reference to the conventional DLS scores and dose–response curves such as shown in Figures 2 and 3. However, this approach would require a unique organism for each drug to be detected. Since the conventional DLS scores yield at best two distinct numbers D_t and M_t, much of the subtle morphological information present in the DLS patterns is ignored. For example, in Figure 6, below, we note that the DLS pattern of bacteria as affected by streptomycin alone is quite different from patterns showing the effects

of the presence of tetracycline. The details of the shape variations of the DLS patterns yield additional and important information about the drugs present. A general mathematical approach to a more detailed analysis of these patterns is given below.

B. Quantitative Analysis of DLS Response. There are obviously many different mathematical formulations by which changes in the DLS patterns may be quantitated and subsequently used to identify and quantitate the drug(s) present. For example, one might take the moments, I_n, of the DLS patterns defined by:

$$I_n = \frac{\int_{\theta_1}^{\theta_2} \theta^n f(\theta)\,d\theta}{\int_{\theta_1}^{\theta_2} f(\theta)\,d\theta} \quad (3)$$

where $f(\theta)$ is the logarithmic DLS pattern and $\theta_1 < \theta_2$ define the range over which the DLS pattern is measured. Although such moments could serve to monitor changes in the shape of $f(\theta)$, they do not directly reflect the periodicity of the scattering pattern. In addition, it must be noted that $f(\theta)$ is an *empirical* function based on empirical data. The DLS measurement process consists of deducing the form $f(\theta)$ by recording the values $f_i(\theta_i)$ at a set of angles $\theta_1 \leq \theta_i \leq \theta_2$. Such measurements are made by the current differential III system by means of a scanner system that records $f(\theta)$ at each 1° increment between 30 and 130°.

Because of debris, bacterial agglomerations, cuvette imperfections, etc., the recorded DLS values are subject to statistical and systematic errors that may often be quite large. We first need, therefore, an analytic representation of $f(\theta)$ based on recorded values each of which is subject to the usual uncertainties of experimental measurement. There is a particular means of achieving such a representation in terms of the experimental data such that the analytical form of the derived function has a minimum least-squares deviation from the data. This consists of an approximation to the data by means of a truncated series of modified Chebyshev polynomials (Lanczos, 1938; Abramowitz and Stegun, 1964; Lanczos, 1956). The 100 data points recorded by the Differential III can be shown (Wyatt, unpublished) to be best represented by the sum:

$$f_n(\theta) = \sum_{m=0}^{n} a_m C_m(x) \quad (4)$$

where C_m is the modified Chebyshev polynomial (Abramowitz and Stegun, 1964), and $0 < x = (\theta - \theta_1)/(\theta_2 - \theta_1) = (\theta - 30)/100 < 1$ and θ is expressed in degrees. For the bacteria of interest, $n = 15$ yields a good representation of the measured DLS pattern in the least-squares sense. A further discussion of the mathematical background relating to the choice of eq 4 is beyond the scope of this paper and the interested reader should refer to the references cited above. Suffice to remark that each of the 15 coefficients a_m is generated in a straightforward manner from the 100 data points recorded. For example, the first coefficient, a_0, is just the average value of the 100 points.

Analysis of DLS data using Chebyshev orthogonal polynomials allows the convenient extraction of morphological information by removing noise and other uninteresting functional variation. The original DLS data for normally growing *S. aureus* (Sabbath) are shown together with reconstructed data containing 5, 10, and 15th degree polynomials in Figure 7.

As shown in eq 2, the Differential III growth displacement term yields a score of 300 for each factor of 10 difference in the scattered intensity. Accordingly, all points on each recorded logarithmic DLS pattern are represented by values between 0 and 1000 (corresponding to a dynamic response of the instrument of $3^1/_3$ orders of magnitude). For purposes of graphical representation, these values are further scaled by a factor of 0.02 to yield a difference of 6 for each order of magnitude difference in the recorded intensity. All DLS protocols are designed to produce results within the aforementioned ranges.

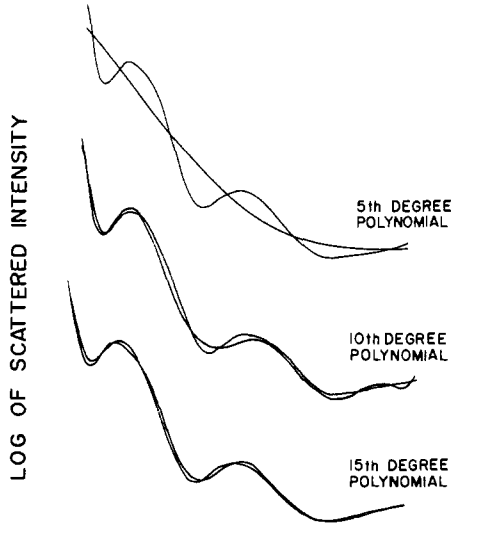

Figure 7. Chebyshev decomposition and partial reconstitution of DLS data from *Staphylococcus aureus* (Sabbath) are shown for 5, 10, and 15 terms.

The representation of the 100 point data sets by the 16 coefficients a_n, $n = 0, 1, \ldots 15$, provides an analytical means by which the changes in the DLS patterns may be followed. As different drugs have different modes of action and cause different types of changes in the amplitude and shape of the DLS patterns, the coefficients a_n may be used to follow such changes and assist in the presumptive identification of the unknown drugs present. Further development of this quantitative approach is certainly called for, especially in reducing the experimental uncertainties of the a_n. Nevertheless, the results of a preliminary examination of this approach are most encouraging as evidenced by the following:

The Chebyshev coefficients of Table III were generated from DLS patterns recorded for *S. aureus* (Sabbath) in the presence of various drugs in a standard growth medium. This organism is resistant to the tetracyclines and streptomycin. All cuvettes have penicillinase (427 IU/mL) and $MgCl_2$ (20 mM) to provide penicillin resistance and augment tetracycline resistance, respectively. All drugs are present at levels of 1 μg/mL except sulfaquinoxaline (5 μg/mL). One cuvette represents I_o, the DLS pattern of the initial inoculum, and two cuvettes contain control suspensions. Variations of a_n values between these two controls represent a fair measure of the types of data-fluctuation induced variations that may be expected. There are several marked differences between the cuvettes containing sulfaquinoxaline and those containing the other drugs or the controls. These are evident by inspection; for example, only the sulfa-containing samples have $a_7 < a_8$. In addition, there are large quantitative differences between the coefficients a_4, a_5, a_9, a_{10}, and a_{11} for sulfa-containing specimens relative to those containing other or no drugs. The selective sensitivity of the test organisms to the sulfas in the presence of some other drugs is thereby clearly demonstrated on a quantitative basis.

SUMMARY, REMARKS, AND RECOMMENDATIONS

This study has demonstrated that the DLS method can provide a fast, sensitive assay method for the detection and

Table III. Chebyshev Coefficients (a_n) for Various Drug Combinations (*S. aureus* (Sabbath))

	n														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Control	10.8	3.35	1.5	−0.115	−0.155	−0.537	−0.169	1.00	0.982	0.054	−0.293	−0.095	0.072	0.046	−0.038	−0.081
Penicillin, 1 μg/mL	10.9	3.43	1.53	−0.071	−0.071	−0.472	0.003	1.14	0.937	−0.048	−0.306	−0.075	0.057	0.011	−0.063	−0.070
Streptomycin, 1 μg/mL	11.0	3.54	1.55	−0.125	−0.093	−0.424	−0.021	1.12	0.912	−0.065	−0.320	−0.070	0.051	0.006	−0.068	−0.072
Chlortetracycline, 1 μg/mL	10.9	3.38	1.44	−0.098	−0.073	−0.487	−0.118	1.01	0.925	−0.017	−0.290	−0.083	−0.058	0.027	−0.050	−0.063
Penicillin + streptomycin, 1 μg/mL each	10.7	3.35	1.54	−0.081	−0.086	−0.480	−0.089	1.07	0.942	−0.014	−0.309	−0.075	0.061	0.025	−0.043	−0.072
Penicillin + chlortetracycline, 1 μg/mL each	10.7	3.37	1.58	−0.083	−0.131	−0.515	−0.085	1.07	0.953	0.004	−0.311	−0.085	0.062	0.020	−0.051	−0.070
Penicillin (1 μg/mL) + sulfa (5 μg/mL)	10.3	3.37	1.8	−0.214	−0.265	−0.493	−0.339	0.752	1.01	0.182	−0.282	−0.116	0.067	0.065	−0.017	−0.062
Streptomycin + chlortetracycline, 1 μg/mL each	10.9	3.29	1.44	−0.085	−0.172	−0.533	−0.162	0.984	0.972	0.039	−0.304	−0.071	0.079	0.047	−0.035	−0.070
Sulfa (5 μg/mL) + streptomycin (1 μg/mL)	10.6	3.28	1.62	−0.078	−0.221	−0.508	−0.368	0.724	0.998	0.210	−0.283	−0.109	0.063	0.057	−0.002	−0.058
I_0 (initial inoculum)	8.06	1.88	1.09	0.036	−0.205	−0.299	0.032	0.585	0.589	0.111	−0.123	−0.065	0.028	0.020	−0.005	−0.026
Control	10.9	3.38	1.51	−0.097	−0.171	−0.527	−0.190	0.942	0.984	0.083	−0.308	−0.095	0.064	0.041	−0.037	−0.065

quantitation of antibiotic residues. The technique provides a report in less than 3 h, shows very good detection limits, has excellent potential for identifying drugs, and even permits a rapid screening for sulfonamides.

Field trials at the USDA APHIS Laboratory confirmed the method's usefulness for rapid negative screening (see Wyatt et al., 1977). These trials also served to underscore the limitations of the Differential III as an assay instrument. We soon learned that correction must be made by the machine for the background scattering level of each sample, since that background may contribute significantly to the observed signal. Important labor reductions over conventional methods were achieved by the further refinement of the tissue press juice extraction process, replacing the time-consuming grinder/extraction techniques. However, sample preparation for DLS may well require more care in regulating temperature, timing, media, and reagent dispensing than required by the plate methods. These controlled procedures introduce some additional hand labor in a manual DLS assay but they can easily be incorporated in an automated assay system. A practical DLS assay system should have a relatively high level of automated sample handling, both to reduce labor costs and to improve the accuracy of the test.

Sample-to-sample noise variations cause problems with reproducibility. Rapid preparation methods may leave occasional pieces of debris in the sample. Such floating particles, or even defects in the plastic cuvettes may on occasion cause a large change in the light scattering levels from otherwise identical specimens. Effective assay instruments incorporating detector arrays rather than a single scanner will permit improved signal averaging and noise detection methods.

Having shown that the DLS method is feasible, it remains as a next objective to delineate the practicability and scope of application of the approach. A basic FDA goal in recent years has been the establishment of possible *antemortem* tests of urine and serum as indicators of latent tissue residues. An extensive program of tissue/urine correlation studies, especially in cooperation with the ongoing work using other assay methods, would yield invaluable data in this regard.

Tissue binding analyses represent a high priority for future work. DLS methods are particularly attractive in this regard since they permit a high degree of automated analyses of large numbers of animal specimens.

Although the DLS methods are highly sensitive and fully automated DLS equipment promises great economies in sample testing, it must be recognized that such methods must eventually be used in combination with other techniques both chemical and biological to yield the types of data required for effective enforcement programs. Not only should field comparisons be made with standard plate methods, but parallel studies with complementary methods such as spore (*Bacillus sterothermophilus*) deactivation and thin film liquid chromatography should be pursued.

In addition to more detailed tissue binding studies, consideration should be given to expanding the types of inhibitory compounds detected. This includes additional antibiotics such as chloramphenicol and cephalothin as well as toxic substances such as pesticides and heavy metals. New screening organisms should be identified and developed by genetic recombination techniques. Chebyshev and related analytical decompositions of the DLS patterns should be studied in detail and new assay algorithms developed and incorporated into new coding sets for detecting and differentiating mixed drugs present in single specimens.

Finally, studies should be made of the application of DLS methods to other regulatory areas relating to food and veterinary products. One area of particular interest is that of mycotoxin detection and identification both in tissues and feed grain. The DLS methods hold exceptional promise in this regard as well as in such diverse areas as vitamin assays in processed foods and pesticide levels in animal tissue, urine, and saliva.

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Laser Light Scattering Bioassay for Veterinary Drug Residues in Food Producing Animals. 3. Screening Bovine Tissues for Drug Residues

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Differential light scattering (DLS) techniques have been applied for the rapid screening of bovine tissues for drug residues. The study was performed with a semiautomated laser light scattering photometer (Differential III) using bovine specimens received by the USDA APHIS Laboratories from their field offices. The results were compared with the standard well diffusion plate methods run in parallel by USDA staff. Of the 172 bovine specimens examined, 31 additional positive tissues were detected (57 by DLS vs. 26 by standard plate assay).

In the previous paper (Wyatt et al., 1977) we have described in detail how differential light scattering (DLS) methods may be applied to the assay of residues in animal tissues. Tissues are squeezed in a gravity activated press. After filtering, the juices are combined with exponential phase bacteria of varying sensitivities, incubated for 2 to 3 h, diluted, and placed in cuvettes for reading in a laser light scattering photometer (Wyatt, 1975). The measured DLS patterns, after correction for tissue background contributions, are then compared with similarly obtained patterns derived from normal, drug-free tissues. Changes in these patterns are then analyzed by means of a mathematical algorithm to yield a score indicative of the effects of the drugs (if any) present.

The objective of the present study was to confirm the practicality of the method as a rapid, negative screening technique. The continuous search for new animal tissue screening methods for antibiotic residues represents an important task for the U.S. Department of Agriculture (USDA), the Food and Drug Administration (FDA), the food producers, and the drug manufacturers themselves. The present study confirms that the laser-based DLS bioassay method can detect all positive animals identified by the conventional plate methods and, in addition, detect residues not detected by the present standard screening methods. These additional residues may be trace heavy metals, pesticides, drugs not generally screened, or other nonspecific bacterial inhibitors.

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SCREENING PROTOCOL AND DLS SCORE

Details of tissue preparation protocols are presented in the companion paper (Wyatt et al., 1977). For the present screening study, frozen samples of bovine liver, kidney, and/or muscle were provided by the Animal and Plant Health Inspection Service (APHIS) Laboratory. Two bacterial strains were used: *Staphylococcus aureus* SS41 and *Klebsiella pneumoniae* SS 886. The complementary sensitivities of these strains to a broad range of antibiotics are described in the companion paper. In addition, we have found in unpublished studies conducted here that *S. aureus* 41 is sensitive to a wide variety of other substances at very low (tenths of micrograms per milliliter or less) concentrations. These include heavy metals, pesticides, antineoplastic drugs (Wyatt et al., 1976), etc.

Prepared tissue juices were divided into three aliquots, one of which was subsequently combined with an exponential phase culture of the *S. aureus*, *Klebsiella*, or an equivalent volume of a pure broth. (The latter mixture would serve as a measure of the tissue background contribution for subsequent DLS calculations.) After incubation, these mixtures were diluted, allowed to equilibrate, and then read on a Differential III (registered trademark of Science Spectrum, Inc.) photometer as has been described previously (Wyatt et al., 1977). All specimens were compared against control juices extracted from equivalent tissues known to be residue-free.