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.

Science Spectrum, Inc., Santa Barbara, California 93101.

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.

Table I. Antibiotic Selective (AS) Specimens

Specimen no.	Kidney			Liver			Muscle		
	Scores	DLS	Plate	Scores	DLS	Plate	Scores	DLS	Plate
713	5, 59	S	–						
716	176, 250	+	+	150, 90	+	+			
717	15, −17	–	–	−46, 8	–	–			
718				22, −2	–	–	9, −10	–	–
719				−14, 28	–	–	65, −14	+	–
720	22, −28	–	–	−56, −180	–	–	57, 3	S	–
721	−34, 16	–	–	−43, −43	–	–	55, −9	S	–
722	24, −38	–	–			–			
723	32, −75	–	–	37, 149	+	–	49, 26	S	–
724	10, −13	–	–	−4, 10	–	–			
725	23, −25	–	+	39, 104	+	–	7, −10	–	–
726	−24, 120	+	–	−168, 15	–	–	52, 13	S	–
727	199, 32	+	+	71, 120	+	+	193, −36	+	–
728	15, 83	+	–	−24, 93	+	–			
729	6, 40	–	–			–	−9, −13	–	–
731	34, 201	+	–	25, 33	–	–			–
732	19, 24	–	–			–			
733	−24, 7	–	–	53, 91	+	–			
734	19, 12	–	–			–	−5, −46	–	–
735	13, 153	+	–	22, 70	+	–			
736			+	48, 69	+	–	60, 23	S	–
737	−58, 41	S	–	9, 14	–	–	28, 22	–	–
738			+	41, 58	S	–	34, 39	–	–
739	56, 58	S	+	22, 92	+	–	12, 15	–	–
740	−7, 48	S	–	−83, 14	–	–	16, 26	–	–
741							−28, −3	–	–
742	82, 49	+	+				64, 16	+	+
743	18, −31	–	–				182, 17	+	+
744						–	−77, −104	–	–
745A							44, −25	S	–
B							−17, 40	S	–
746							69, 35	+	–
747A							−27, 44	S	–
B							−88, 101	–	–
748							−14, 26	–	–
749							−39, −78	–	–
750	159, −12	+	+	−81, −25	–	–	−43, −189	–	–
751							56, −29	S	–
752							−41, −52	–	–
753	−16, −26	–	–	95, 13	+	+	65, 89	+	–
754	−25, −98	–	–	−38, −51	–	–	−11, 16	–	–
755				−122, 64	+	–	4, 26	–	–
756	−20, −43	–	+				10, −15	–	–
757	19, 54	S	–						–
758	8, 33	–	–						–
766	99, 32	+	–				49, 0	S	–
768	−25, 69	+	–	153, −21	+	–			
771	2, 28	–	–	−36, −35	–	–			
772	−31, −48	–	–	−8, 98	+	–			
773	−40, 8	–	–	74, 129	+	+			
774	−5, 3	–	+	44, 82	+	–			
775	−86, −61	–	–	55, 118	+	–			
776	−24, −2	–	+	45, 72	+	–			
778	−24, −21	–	–	4, 25	–	–			
790	−37, −41	–	+	17, 28	–	–			
795	151, 7	+	+	254, 95	+	+			

The DLS patterns of all specimens were measured with respect to the control tissue blanks. A screening score, S, was then defined in terms of the various DLS displacement scores (Wyatt et al., 1977) as follows:

$$S = 300 \log \frac{10^{(D_{bc}/300)} - 10^{(D_{jc}/300)}}{10^{(D_{bt}/300)} - 10^{(D_{jt}/300)}} \quad (1)$$

where D_{bc} = displacement of control juice plus bacteria relative to control blank, D_{jc} = displacement of control blank relative to itself (= 0 in the absence of noise), D_{bt} = displacement of test juice plus bacteria relative to control blank, and D_{jt} = displacement of test juice blank relative to control blank. The screening score, S, provides a precise measure of the growth of assay bacteria in the test sample relative to control growth.

The Differential III records the DLS patterns in a logarithmic mode, as explained previously (Wyatt et al., 1977). Thus the complexity of eq 1 is but a reflection of the conversion back to linear forms from which juice backgrounds may be subtracted and thence back to the usual logarithmic form. Equation 1 yields a factor of 300 for each tenfold increase of the bacteria exposed to control (drug-free) juices relative to those exposed to test juices.

The DLS bacterial bioassay technique permits the use of other indicators of response in addition to bacterial growth. Changes in bacterial structure and size distribution have been found effective for certain assay procedures (Wyatt et al., 1977). These morphological measures of response can also be incorporated in a practical DLS assay system though they were not used in the present study.

Table II. Antibiotic Objective (AO) Specimens (Primarily Kidney Tissues[a])

Specimen no.	Kidney			Specimen no.	Kidney		
	Scores	DLS	Plate		Scores	DLS	Plate
25	−40, −8	−	−	73	−19, 19	−	−
26	21, 14	−	−	74	−26, −5	−	−
28	28, 43	S	+	75	−28, 14	−	−
30	13, 48	S	−	76	−126, −41	−	−
44	−1, 101	+	−	77	−64, 1	−	−
45	57, 51	S	−	78	−28, 8	−	−
46	5, −20	−	−	79	9, 51	S	+
47	76, 15	+	+	80	−53, 76	+	−
48	1, 113	+	−	81	−25, −35	−	−
49	46, 77	+	+	82	−56, −137	−	−
50	18, 84	+	−	83	−64, 11	−	−
51	−10, 78	+	−	84	−39, 13	−	−
52	7, 44	S	−	85	−20, 13	−	−
53	1, 26	−	−	86	−21, −6	−	−
54	1, 199	+	+	87	−22, −27	−	−
55	−20, 88	+	−	88	−36, 9	−	−
56	66, −22	+	−	90	−25. 5	−	−
58	−10, 27	−	−	91	−43, −77	−	−
59	26, −131	−	−	92	−67, −30	−	+
60	5, 33	−	−	93	−84, −34	−	−
62	30, −41	−	−	94	−58, −28	−	−
63	−14, 3	−	−	95	−45, −28	−	−
64	3, −26	−	−	96	−84, −36	−	−
65	−7, −9	−	−	98	−50, −49	−	−
66	3, 35	−	−	99	−63, −72	−	−
67	−35, −44	−	−	100	43, −15	S	−
68	−37, −43	−	−	101	−99, −67	−	−
69	2, −27	−	−	102	40, 25	−	−
70	−10, 6	−	−	103	−6, −25	−	−
71	−16, −20	−	−	105	−38, −34	−	−
72	−6, −8	−	−	104	−32, 17	−	−

[a] Animals 79 and 92 were also studied via liver tissues. For animal 79, the positive DLS scores were 92 and 74; the plate result was negative. For animal 92, the positive DLS scores were 55 and 99; the plate result was positive.

Because of noise arising from unfiltered tissue debris, bacterial agglomerations, and/or protein precipitations, the fluctuations of these scores are quite large. The effect of these optical noise sources can be greatly reduced by improved signal averaging methods. The two bacterial strains (*S. aureus* 41 and *Klebsiella pneumoniae* 886) tested against the juices were sensitive to certain drugs at levels below maximum tolerances allowed by FDA regulation. Thus a score indicative of a positive tissue was experimentally established for screening purposes to be greater than 60. Specimens with scores below 40 were classified as negative tissues, while those between 40 and 60 were categorized as suspect or intermediate.

RESULTS

A typical assay was completed within 2.5 h after combining the tissue extract with the assay organisms. DLS assays were performed for 172 tissues, considered representative of those analyzed by the APHIS Laboratory. Such tissues are generally of two classes: antibiotic selective (AS) tissues, i.e., those picked subjectively by the USDA inspectors because of injection sites, pathological lesions, etc.; and antibiotic objective (AO) tissues, i.e., from animals selected at random by inspectors for general screening studies. When kidney and liver tissues from the same animals were assayed by DLS, *all* residue-containing animals picked up by the APHIS Laboratories were confirmed positive by DLS; however, for six cases the liver rather than the kidney tissues were found to produce a much greater DLS assay response. Not counting these six cases, an additional 31 tissues had elevated DLS responses due to unidentified causes (e.g., heavy metals, antibiotics not conventionally screened, pesticides, etc.) were detected (57 positive by DLS assay vs. 26 by standard plate bioassay) and 18 additional suspect or intermediate tissues found. Many of these "extra" tissues were very "hot" by DLS test (scores in excess of 100). Further tests of such samples using other methods are planned to identify the residues present. The highest percentage (35%) of additional positive or suspect tissues were found among AS (antibiotic selective) tissues, as expected, whereas only 17% additional residue tissues were detected among the AO (antibiotic objective) samples. These most likely represent residues below established legal tolerances. By chance, during the period of these tests, the AO samples came primarily from animals that may have been treated for mastitis. Tables I and II contrast the results of the DLS and standard well diffusion plate tests performed by USDA personnel for AS and AO tissues, respectively.

No plate assay data were available for AO liver and muscle tissues corresponding to plate-negative kidneys as the kidneys were used as the primary screening sample. A careful study of Tables I and II, however, does disclose (even for this limited sample set) that negative kidney results do not always correspond to negative liver or muscle (usually injection site) results and vice versa. The decision by USDA to use kidney as the target assay tissue is based on the best toxicological and bioassay information available. For the most part the decision is still valid, except for several recently introduced therapeutic agents that have an affinity for liver tissue. Since these drugs are expensive, they are infrequently used; this does not preclude the possibility that this will not change in the future. The USDA is closely following the situation and will make adjustment as required.

The pair of numbers listed opposite each sample number under the heading "scores" corresponds to the DLS screening scores (per eq. 1) for the assay organism *S.*

Table III. DLS Score Summaries[a]

Sample type	No. samples	Result		Average DLS score[b]
		DLS	Plate	
AO kidney	45	−	−	−30 ± 31; −18 ± 38
	3	+	+	76 to 199
	7	+	−	86 ± 16
	1	−	+	−67; −30
AS kidney	19	−	−	−5 ± 30; −15 ± 38
	5	+	+	168 ± 62
	5	+	−	128 ± 51
	5	−	+	−12 ± 21; −21 ± 21
AS liver	13	−	−	−29 ± 53; −11 ± 58
	5	+	+	150 ± 62
	12	+	−	97 ± 30
	0	−	+	None
AS muscle	18	−	−	−13 ± 34; −30 ± 58
	2	+	+	68 to 182
	4	+	−	65 to 193
	0	−	+	None

[a] The data shown in Tables I and II are summarized below. DLS scores below 40 are negative, DLS scores above 60 are positive. [b] Based on five samples or more.

aureus 41 and *K. pneumoniae* 886, respectively. Note in the case of positive tissues, both organisms do not necessarily yield "positive" scores. Because of their different sensitivities, the responses of these organisms could be used to deduce some presumptive drug identifications, though none were attempted under the current study.

Table III summarizes most of the data of Tables I and II. Averages are presented when 5 or more tissues were available; otherwise the DLS growth score ranges are shown.

The additional positive and suspect tissues detected, even in the small sample set examined, are of particular interest. Although such positive tissues may contain drug levels well below conventionally acceptable thresholds, the *detection* of such tissues will represent a useful source of information both to USDA and FDA in evaluating the effectiveness of current residue control practices. In addition, the bacterial assay strains used with the system seem more sensitive to a wider variety of antibiotics and many other types of toxic agents than the conventional strains. Positive tissues could contain pesticides or other nonspecific substances whose persistence in the food supply may be of considerable interest and importance. Since the DLS assay procedure will be capable of screening significantly greater numbers of tissues, the additional positive results confirmed by other methods if and/or when available might well be used to identify producers having residue problems of which they themselves are unaware. Increased AO screenings of *all* tissue types should result in a better means for identifying chronic violators of Federal residue standards as well as potential trouble areas. It must be pointed out, however, that FDA established tolerances on residues in edible tissues are based on toxicological data with a built in safety factor for humans. Therefore, increased residue findings are at present of little value until FDA learns from new toxicological data that the established tolerances are hazardous to human health. At this point, FDA may either revise the tolerances or even ban the use of the therapy entirely.

The six positive plate results not confirmed by DLS lend further weight to the usefulness of testing *both* kidney and liver tissues. All six were kidney tissues and of these, only five had parallel bioassays performed on livers (AS 725, 774, 776, 790, and AO 92). The corresponding livers of four of these specimens were strongly positive by DLS (except for AS 790) and, therefore, only one of the positive animals could have passed a rigorous DLS assay in which *both* kidney and liver were assayed. Specimen AS 790 seemed an anomaly and may well have been negative. Time did not allow a bioassay and DLS rerun for this tissue. In addition, note that AS kidney tissue 738 was not available for DLS assay and AS liver tissue 766 was lost because of a faulty assay culture. Nevertheless, the animals from which these specimens were obtained obviously contained residues, since DLS picked up suspect or positive liver and kidney tissues, respectively, for the two cases. Plate bioassay positive kidney tissues AS 739 and AO 79 showed high suspect DLS scores, while the corresponding liver tissues both confirmed a residue-positive animal.

CONCLUSIONS

The DLS bioassay technique has been shown to be a rapid and useful method for screening animal tissues for antibiotic residues as well as detecting the presence of nonspecific antibacterial substances. DLS results were available within 4 h while plate methods took 1 or more days. The simplicity of sample preparation in itself represents a major source of cost savings. Even without identification or quantitation, the DLS screening procedure can reduce dramatically the number of tissues for which full analytical testing is required. A more fully automated form of the DLS assay promises to provide a sensitive technique with fast turn around for routine screening of large numbers of samples.

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