JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLES

Determination of Anthraquinone in Technical Material, Formulations, and Lettuce by High Performance Liquid Chromatography

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Foraging on lettuce seeds and seedlings by horned larks (*Eremophila alpestris*) causes millions of dollars in losses to the California lettuce crop annually. Anthraquinone (AQ; 9,10-anthracenedione) has been shown to deter pest birds from consuming the seeds and seedlings of several plant species and was evaluated as a repellent to horned larks when applied to lettuce seedlings. A set of analytical methods using simple liquid extraction followed by high-performance liquid chromatography analysis were developed for the quantitation of AQ as technical material, as an active ingredient in a commercial formulation, and as a residue in lettuce plants. The methods were easy, reliable, and repeatable. AQ recoveries from control formulation fortified to concentrations of either 24 or 600 mg g^{-1} were 99 (±1.2%) and 98% (±1.2%), respectively, with a control formulation method limit of detection (MLOD) of 0.50 mg g^{-1}. Control lettuce tissues from three growth stages were AQ-fortified to concentrations of 0.50 and 500 μg g^{-1}. The resulting AQ recoveries for the two fortification levels were 99 (±8.5) and 89% (±1.5%) for 11 day old seedlings, 95 (±2.6%) and 86% (2.1%) for 16 day old plants, and 92 (±1.4%) and 93% (±1.1%) for adult head lettuce cover leaves, respectively. The MLODs for the same three lettuce tissues were 0.055, 0.058, and 0.077 μg g^{-1}, respectively. These methods were used to quantify AQ residues from field-grown, treated lettuce and associated fortified quality control samples.

KEYWORDS: Anthraquinone; 9,10-anthracenedione; CAS No. 84-65-1; lettuce; *Lactuca sativa*; horned lark; *Eremophila alpestris*; HPLC

INTRODUCTION

Lettuce (*Lactuca sativa* L.) is a significant field crop in California, with 3.3 million tons harvested on over 85 000 hectares, generating revenues of more than one billion dollars in 1999 (*1*). Foraging by horned larks (*Eremophila alpestris*) on newly planted lettuce seeds and seedlings causes millions of dollars in losses annually (*2*). Recently, the National Wildlife Research Center (NWRC, a USDA research facility in Ft. Collins, CO) investigated the application of a variety of nonlethal, repellent compounds to protect newly emerged lettuce seedlings. One compound that yielded encouraging results was anthraquinone (AQ; 9,10-anthracenedione).

AQ occurs naturally in many plant species and is used in the dye and paper pulping industries (*3*). Physically, AQ absorbs UV light with absorbance maxima at 254 (λ_1), 273 (λ_2), and 325 nm (λ_3). It is insoluble in water and has limited solubility in most common solvents, with a maximum solubility in chloroform of 0.61 g/100 g at 20 °C (*4*). AQ also has a low toxicity, with an oral LDL_0 (rat) of 15 000 mg kg^{-1} (*5*) and a dermal LDL_0 (rat) of >1000 mg kg^{-1} (*6*).

Initial experimental evidence of AQ efficacy as a bird repellent was described in a German patent application covering the use of a variety of anthraquinones as bird feeding deterrents (*7*). Crows (*Corvus* spp.) that were offered wheat treated with a 0.2% mixture of 25% AQ/75% talcum (as carrier) ate only 2.5% of the bait while crows given control wheat (0.2% talcum only) consumed 100% of the bait. This led to the development of the first commercial AQ formulation, Morkit. A subsequent U.S. patent (*8*) allowed for the importation and distribution of Morkit in the U.S. Extensive testing during the early to mid-

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10.1021/jf0113878
Published on Web 05/16/2002

1950s showed significant reduction in bird-related loss to pine seeds treated with Morkit (*9*, *10*), 99.9% pure synthetic AQ (*9*, *11*), and 96% pure crude AQ (*11*). Subsequent experiments also demonstrated reduced consumption of crude AQ-treated rice seed in several bird species (*12*). However, the importation of Morkit was discontinued in 1956 and the product was never registered as a bird repellent. Recently, the availability of new commercial AQ formulations has prompted renewed research interest in AQ as a feeding deterrent in birds. Treatment with one such AQ formulation significantly reduced consumption of rice seed by male red-winged black birds (*Agelaius phoeniceus*) and female boat-tailed grackles (*Quiscalus major*; *13*). Application of a different AQ formulation to millet seeds and turf markedly decreased intake by brown-headed cowbirds (*Molothrus ater*) and Canada geese (*Branta canadensis*), respectively (*14*).

Flight Control (Environmental Biocontrol International [EBI]; Wilmington, DE), a formulation containing 50% AQ (a.i.), surfactants, and water, was field-tested as a repellent on newly emerged lettuce seedlings in the San Joaquin Valley of California (*15*). To support the field research, analytical methods were developed, validated, and used to assess AQ concentrations in technical material, in the formulation, and in the lettuce crop at various stages of growth. Expected AQ concentrations ranged over 4 orders of magnitude (0.50−500 μg g^{-1}).

Several analytical methods for AQ utilizing high-performance liquid chromatography (HPLC) have been reported, primarily for quantification of the compound in paper production-related matrixes such as wood, pulp, and pulping liquors (*16*−*20*). Primus et al. developed an HPLC method for AQ determination in formulated rice seed and surface water (*21*). This paper describes methods for the preparation and HPLC analysis of AQ technical material and formulation samples, as well as a simplified solvent extraction and HPLC gradient elution/cleanup method for AQ residues in small (0.5 g) quantities of lettuce from various growth stages.

MATERIALS AND METHODS

Sample Preparation. *Technical Material.* A 10 mg portion of the AQ technical material (ChemService, Westchester, PA; 99% purity) was dissolved in chloroform (GC^2 grade, Burdick & Jackson, Muskegon, MI) to yield a final concentration of 0.40 mg mL^{-1}. A 0.50 mL aliquot of the 0.40 mg mL^{-1} AQ/chloroform solution was then diluted with methanol (Omnisolv grade, EM Scientific, Gibbstown, NJ) to a final concentration of 0.040 mg mL^{-1}, filtered through a 0.45 μm Teflon syringe filter into an amber HPLC vial, and capped.

Formulation. Prior to sampling, the Flight Control formulation was homogenized using a mechanical shaker (Equalpoise, model 6550, $2^3/_8$ stroke, Eberbach, Ann Arbor, MI) and shaken at ≈175 strokes min^{-1} for 20 min. A 0.100 g portion of the formulation was accurately weighed into a 25 mL glass tube, 15.0 mL of chloroform was added, and the sample was vortex-mixed. The sample was further dissolved/mixed in a room temperature ultrasonic bath (Bransonic 32, Branson Ultrasonics Corp., Danbury, CT) for 40 min. The chloroform extract was then transferred to a 25 mL volumetric flask along with two additional 2.5 mL chloroform rinses of the 25 mL glass tube. The combined extracts were brought to volume and mixed. A 0.500 mL aliquot of the extract was diluted 1:20 with methanol, filtered through a 0.45 μm Teflon syringe filter into an amber HPLC vial, and capped.

Lettuce. Field-grown lettuce seedlings/plants were collected immediately prior to treatment with Flight Control, immediately posttreatment, and on 3, 6, 12, and 50 days posttreatment. The entire plant was uprooted, and the roots were removed. Plants were placed in a plastic bag, refrigerated at 4 °C for ≈4 h, and then transferred to a freezer (−10 °C) and shipped overnight to the laboratory where samples were stored at −14 °C until analyzed. Immediately prior to extraction and analysis, plants were cryogenically homogenized (*22*, for all sampling times except 50 days posttreatment). For 50 day posttreatment plants, the outer two cover leaves were removed and cryogenically homogenized. Approximately 0.5 g of frozen, powdered lettuce was transferred to a 25 mL screw top glass tube with a 13 mm blue-faced white silicone septum (Supelco, Bellefonte, PA) in the cap. Chloroform (10.0 mL) was added to the sample, which was then vortex-mixed, shaken at high speed (≈175 strokes min^{-1}) for 15 min, vortex-mixed, sonicated for 15 min, and vortex-mixed again. Following a 15 min centrifugation (≈2000 rpm; Centrific 225, Fisher Scientific, Pittsburgh, PA), the chloroform layer was transferred to a 10 mL glass screw top tube. A 1.00 mL aliquot of the chloroform extract was transferred to a 1.00 mL volumetric tube and blown to dryness under N_2 at room temperature. The dried sample was then reconstituted in 0.550 mL of acetonitrile (Optima grade, Fisher Scientific, Fairlawn, NJ), vortex-mixed, sonicated for 15 min, brought to 1.00 mL with water, and vortex-mixed again. The sample was filtered through a 0.45 μm Teflon syringe filter into an amber HPLC vial and capped.

Table 1. HPLC Gradient Timetable for AQ Elution and Column Cleanup[a]

time (min)	%A	%B	flow (mL min^{-1})
0.00	100	0	1.0
13.00	100	0	1.0
13.50	0	100	1.0
16.90	0	100	1.0
17.00	0	100	1.5
21.50	0	100	1.5
21.60	0	100	1.0
22.00	100	0	1.0
30.00	100	0	1.0

[a] Where: Channel A: 55% acetonitrile/45% water, premixed; channel B: 100% acetonitrile.

Chromatographic Conditions. *Technical Material/Formulation.* Extracts were analyzed using an HP 1090 liquid chromatograph (Agilent Co., Sunnyvale, CA) equipped with a diode array detector (UV at 325 nm); column, Keystone (Bellefonte, PA) ODS/H 250 mm × 4.6 mm i.d., 5 μm, 1.5 mm × 4.6 mm i.d. guard column; oven, 35 °C; mobile phase, 80% methanol/20% water, isocratic; flow rate, 1.0 mL min^{-1}; injection volume, 10 μL; run length, 13 min. The elution time of AQ was approximately 6 min.

Lettuce. Lettuce extracts were analyzed using an HP 1090 liquid chromatograph equipped with a HP 1050 variable wavelength detector (UV at 254 nm); column, Keystone ODS/H, 250 mm × 4.6 mm i.d., 5 μm; 1.5 × 4.6 mm i.d. guard column; oven, 40 °C; mobile phase, isocratic elution followed by gradient cleanup, see **Table 1**; injection volume, 100 μL; run length, 30 min. The elution time of AQ was approximately 13 min.

Selectivity, Bias and Repeatability, and Method Limit of Detection (MLOD). *Technical Material.* Five ≈10 mg portions of AQ were weighed into a 25 mL volumetric flask and prepared as previously described.

Formulation. Because a control formulation (all ingredients except AQ) of Flight Control was unavailable from the manufacturer, deionized water was used as a substitute. Five replicates of control formulation and seven replicates each of control formulation fortified at one of two AQ concentrations (24 and 600 mg g^{-1}) were prepared and analyzed.

Lettuce. Method validation was performed on control lettuce of three specific ages, which represented broader growth stages: (i) greenhouse-grown 11 day old seedlings (small two-leafed plants), (ii) field-grown 16 day old seedlings (larger 4−6-leaved plants), and (iii) cover leaves from head lettuce obtained at a local grocery store (representing both preharvest and harvested plants). Plants were processed as previously described. Three sets of seven 0.5 g replicates from each age group were weighed and fortified to one of three AQ concentrations: (i) 0.0 (matrix blank), (ii) 0.50, or (iii) 500 μg g^{-1}. Each fortified sample was allowed to stand for 15 min and then extracted and analyzed using the procedures previously described.

The MLOD was defined as the AQ concentration required to generate a chromatographic response 3× the baseline noise (measured peak-to-

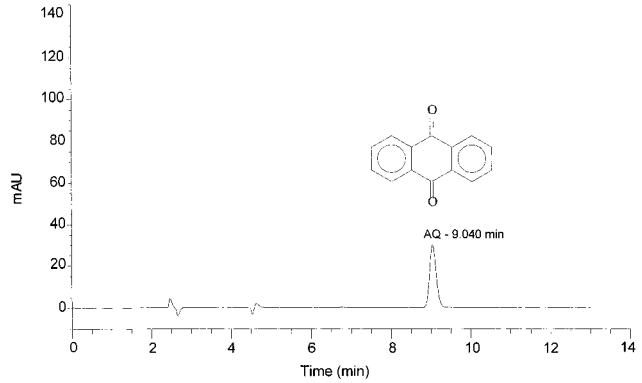

Figure 1. Anthraquinone working standard (25.3 μg mL^{-1}).

peak) found in control formulation or lettuce matrixes at the retention time of AQ. Five replicates of the control formulation and seven control formulation samples fortified to a mean concentration of 24 μg g^{-1} were used to assess the formulation MLOD. Seven replicates each of control tissue and control tissue fortified to an AQ concentration of 0.50 μg g^{-1} were used to assess the MLOD for each lettuce growth stage.

RESULTS AND DISCUSSION

Linearity. Linear regression analysis was performed on the chromatographic response data (*23*). Two independent sets of six AQ standard solutions ranging in concentration from 1.18 to 179 μg mL^{-1} were used to assess standard linearity for both technical material and formulation analyses.

Regression analysis yielded an r^2 of 0.9998, a *y*-intercept of 4.002 ($\pm$4.638) (HO: $y_{int} = 0$, $p = 0.3976$), and a slope of 14.932. A log vs log plot of these data gave an r^2 of 0.9996 and a slope of 0.994 871. Taken together, these results indicated a highly linear and proportional relationship between AQ concentration and chromatographic response and justified the use of a single point calibration standard (25.0 μg mL^{-1}) for subsequent AQ technical and formulation analyses. Two independent sets of eight AQ standards ranging in concentration from 0.0124 to 25.0 μg mL^{-1} were used to assess standard linearity for lettuce analysis. Regression analysis yielded an $r^2 = 0.9993$, a *y*-intercept of 75.435 352 ($\pm$50.263 841) (HO: $y_{int} = 0$, $p = 0.1439$), and a slope of 1136.007. A log vs log plot of these data yielded an $r^2 = 0.9999$ and a slope of 0.989 801. A linear and proportional relationship existed between AQ concentration and chromatographic response, and a single point calibration (1.0 μg mL^{-1}) was used for all subsequent tissue analyses.

Selectivity, Bias and Repeatability, and MLOD. *Technical Material/Formulation.* The mean recovery value for the five AQ technical material replicates was 96.4% ($\pm$0.52%). A chromatogram of the AQ working standard (25.3 μg mL^{-1}) is illustrated in **Figure 1**.

No coeluting peaks were found at the retention time of AQ in any of the control formulation blanks. Mean AQ recoveries for the formulation controls fortified at 24 and 600 mg g^{-1} were 99.0 ($\pm$1.2%) and 98.0% ($\pm$1.2%), respectively.

The water control formulation MLOD was 0.50 mg g^{-1}. Chromatograms of a control formulation sample and a second control formulation sample fortified to an AQ concentration of 594 mg g^{-1} are shown in **Figure 2a**,**b**, respectively. Analysis of a triplicate sample of Flight Control yielded an AQ concentration of 50.1% ($\pm$0.3; target value, 50%), and a chromatogram of a Flight Control formulation sample is included as **Figure 2c**.

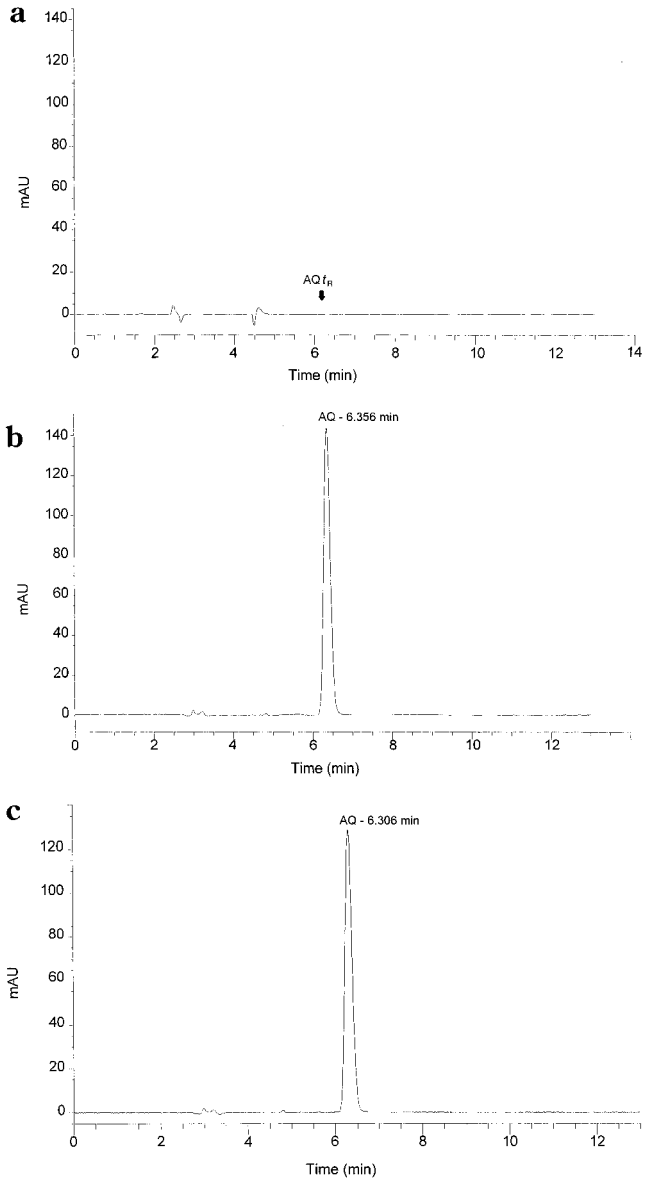

Figure 2. (**a**) Control formulation sample. (**b**) Fortified (594 mg g^{-1}) control AG formulation. (**c**) Flight control formulation (50% AQ nominal).

Table 2. Percent Recoveries from AQ-Fortified Control Lettuce Samples Analyzed during Method Validation and Concurrently with Field-Grown Lettuce Residue Analysis[a]

AQ (μg g^{-1})	11 day old seedlings	16 day old seedlings	cover leaves
	Method Validation ($n = 7$)		
0.50	99 (8.5)	95 (2.6)	92 (1.4)
500	89 (1.9)	86 (2.1)	93 (1.1)
	Quality Control Samples		
$\approx$0.50	101.1 (9.4), $n = 8$	98.6 (6.4), $n = 6$	84.7 (3.8), $n = 6$
$\approx$500	86.1 (1.9), $n = 8$	87.3 (2.6), $n = 7$	94.5 (7.5), $n = 6$

[a] Values in parentheses are standard deviations.

Lettuce. A very small peak was detected at the AQ retention time in one replicate of both the 11 day old and 16 day old lettuce seedlings and in two samples of head lettuce cover leaves, but this response was below the MLOD in all cases. The MLODs for the 11 day old seedlings, 16 day old seedlings, and cover leaves were 0.055, 0.058, and 0.077 μg g^{-1}, respectively. AQ recoveries for both the 0.50 μg g^{-1} fortification

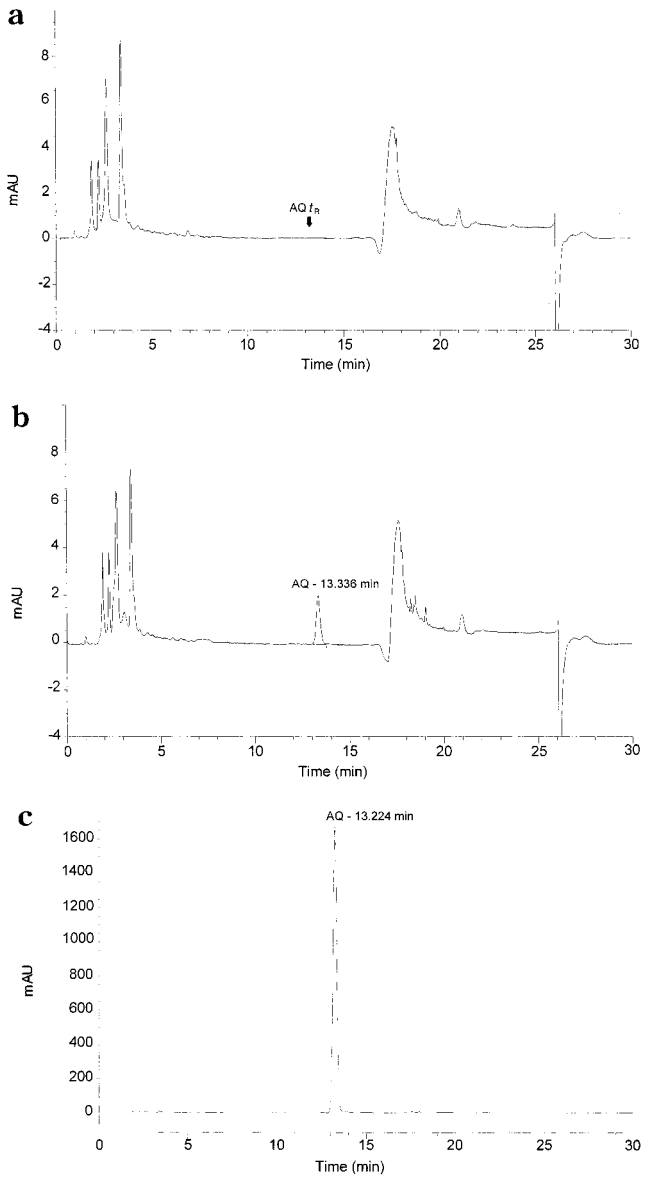

Figure 3. (**a**) Eleven day old lettuce control sample. (**b**) Eleven day old fortified control lettuce seedlings (0.050 μg g^{-1}). (**c**) Eleven day old fortified control lettuce seedlings (500 μg g^{-1}).

and the 500 μg g^{-1} fortification of 11 day old seedlings, 16 day old seedlings, and cover leaves are shown in **Table 2**. AQ recoveries were excellent, exceeding 84% in all cases. Chromatograms of 11 day old control lettuce tissue unfortified and fortified to concentrations of 0.50 and 500 μg g^{-1} are illustrated in **Figure 3a**−**c**, respectively. Additional quality control fortification samples were run concurrently with field-collected lettuce samples, and the resulting recovery data are also shown in **Table 2**. AQ recoveries for equivalent laboratory validation and quality control fortification samples were highly comparable and ranged from 84.7 to 101.1%. Standard deviations for all lettuce matrixes were <10%, with 11 day old lettuce seedlings consistently the most variable matrix when fortified to the 0.50 μg g^{-1} concentration. Analysis of over 60 control- and AQ-treated field samples yielded AQ residues ranging from undetectable to 665 μg g^{-1}.

The combination of isocratic analyte AQ elution immediately followed by a column cleanup solvent gradient allowed for simplified sample preparation without time- and solvent-consuming sample cleanup. During method development, AQ adsorption to and subsequent contamination from stock cap liners was a serious and time-consuming problem. This was alleviated by the use of 13 mm blue-faced white silicone septa inserts.

General. It is frequently difficult to acquire control formulations when developing methods for the analysis of commercially available products, due largely to proprietary ingredient or batch production constraints. A method developer is often left having to use "the next best thing". The substitution of water for an actual control formulation in the context of the methods described here was appropriate, given that Flight Control is an aqueous formulation. Chromatograms from samples of AQ-fortified control formulation and the actual Flight Control commercial formulation (**Figure 2b**,**c**, respectively) were virtually identical.

In contrast to the optimal 254 nm wavelength used for HPLC analysis of lettuce AQ residues, analysis of AQ technical material/formulation samples utilized the λ_3 (325 nm) wavelength, which provided additional analytical selectivity. Any loss of sensitivity was offset by the elevated AQ concentrations in the technical/formulation samples. The use of chloroform as an extraction solvent maximized AQ extraction solubility, and the HPLC gradient cleanup step following AQ elution in lettuce residue analysis reduced preanalysis sample preparation.

CONCLUSIONS

The analytical methods described in this paper are simple, reliable, and repeatable and can be easily applied even when sample quantity is limited. Fortified control formulation samples yielded high (≥98%) recoveries with correspondingly low standard deviations (±1.2%). AQ recoveries from fortified lettuce control samples were more variable but exceeded 84% in all cases, with the majority of sample replicates yielding standard deviations less than ±3%. Additionally, the simplified lettuce sample cleanup procedure allowed minimum sample preparation while still yielding acceptable chromatographic sensitivity. The set of methods presented here allow a complete analysis of AQ in commonly encountered forms such as technical material, as formulation, and as residue in a field crop. The latter may become more common as the effectiveness of AQ as a nonlethal, nontoxic feeding deterrent to birds is demonstrated and its use for that purpose increases.

ACKNOWLEDGMENT

We thank Doreen Griffin and Carol Furcolow for their assistance.

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Received for review October 19, 2001. Revised manuscript received February 15, 2002. Accepted February 15, 2002. Use of trade names does not constitute endorsement by the United States Department of Agriculture.

JF0113878