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Analysis of CoQ_{10} in Cultivated Tobacco by a High-Performance Liquid Chromatography−Ultraviolet Method

Michael R. Stiff, Arthur K. Weissinger, and David A. Danehower*

Department of Crop Science, North Carolina State University, Raleigh, North Carolina 27695-7620, United States

ABSTRACT: Coenzyme Q (CoQ) is a naturally occurring lipid-soluble quinone that performs multiple functions in all living cells and has become a popular antioxidant supplement, a coadjuvant in the treatment of heart disease, and the object of study for treating neurodegenerative disorders. Although there are many tools for CoQ analysis of microbial and animal samples, there have been relatively few reports of methods for CoQ analysis of green plants. This work describes a method for the routine analysis of coenzyme Q_{10} in green leaf tissue of cultivated *Nicotiana tabacum* (tobacco) using high-performance liquid chromatography (HPLC) with UV detection. The method was applied to the analysis of CoQ_{10} in *N. tabacum* 'KY14' leaves at different stalk positions representing young lanceolate to senescing leaves, and it was found that CoQ_{10} increased as leaf position changed down the stalk from 18.69 to 82.68 μg/g fw. The method was also used to observe CoQ_{10} in *N. tabacum* 'NC55' and *N. tabacum* 'TN90LC' leaves over time, finding that CoQ_{10} leaf content remained relatively stable from 3 to 6 weeks but increased in both cultivars at 8 weeks. This method will likely be useful in the analysis of CoQ_{10} in the green leaves of other plant species.

KEYWORDS: ubiquinone, coenzyme Q, tobacco, HPLC

INTRODUCTION

Coenzyme Q (CoQ) is a lipid-soluble quinone with multiple cellular functions. It is an integral part of mitochondrial energy production and a membrane-soluble antioxidant. In plants, CoQ may play a role in pyrimidine biosynthesis[1] and branched-chain amino acid catabolism.[2,3] CoQ also functions in conjunction with the alternative oxidase (AOX) to manage oxidative stress and may be involved in cellular signaling during the induction of systemic resistance to tobacco mosaic virus (TMV) in tobacco.[4−6]

CoQ_{10} (Figure 1) has become a popular antioxidant supplement and coadjuvant in the treatment of heart disease as well as the object of study in the treatment of a number of other diseases such as Parkinson's and Alzheimer's.[7] It has also become a popular lipid-soluble antioxidant in cosmetics.[8]

The biological, medicinal, and commercial significance of CoQ makes the study of CoQ biosynthesis important. CoQ biosynthesis is well studied in *Escherichia coli*[9,10] and yeast;[11] however, it is little understood in plants. The development of fast and reliable tools for the analysis of CoQ_{10} is necessary to promote a better understanding of its various biological functions, evaluate novel sources, and study its biosynthesis. Although there are many tools for CoQ analysis of microbial and animal sources, such tools have been particularly limited for the analysis of plant materials.

This work describes the development of a rapid method for the extraction and routine analysis of coenzyme Q_{10} in green leaf tissue of cultivated *Nicotiana tabacum* (tobacco) using high-performance liquid chromatography with ultraviolet detection (HPLC-UV). The method was used to quantify the levels of CoQ_{10} in *N. tabacum* leaves at different stalk positions and over time in a greenhouse. This method should prove useful in the analysis of CoQ_{10} in other plant species.

MATERIALS AND METHODS

Plant Materials. Seeds for *N. tabacum* 'NC55' and *N. tabacum* 'TN90LC' were generously provided by Dr. Ramsey Lewis of the North Carolina State University Department of Crop Science. Seed for the tobacco cultivar KY14 was provided by Dr. Arthur K. Weissinger of the same institution.

Growth Conditions for Plants and CoQ_{10} Sampling Plan by Experiment. *CoQ_{10} in Leaves at Different Stalk Positions.* A population of KY14 ($n = 5$) was grown in the greenhouse under ambient temperature and 16 h day length. Plants were watered once per day and given 20−20−20 N−P−K liquid fertilizer (350 ppm N) once per week. The plants were sampled after 14 weeks in the greenhouse when they reached the full flowering stage of development. Lanceolate leaves were approximately 14 cm in length. The other stalk positions investigated were positions 5, 7, and 14 leaves from the first leaf at the apex measuring ∼2.54 cm.

CoQ_{10} over Time in the Greenhouse. Populations of NC55 and TN90LC (n = 11 for each) were grown in the greenhouse under the same watering and fertilization regimen as described for leaves at different stalk positions. The first leaf >27 cm in length from leaf axil to leaf tip (usually four to five leaves below the first leaf at the apex measuring ∼2.54 cm) was harvested and placed on ice prior to transportation to the laboratory for sample preparation. Plants were sampled at 3−8 weeks.

CoQ_{10} Analysis by HPLC-UV. *Sample Preparation and Analysis.* A 1.0 cm diameter (50−100 mg fw) leaf disk was sampled, and the leaf veins, if present, were removed. The remaining lamina was weighed and frozen in liquid nitrogen prior to extraction. Our extraction method was adapted from that of Ohara et al.[12] Leaf tissue was homogenized twice for 5 s in a Silamat S5 amalgamator (Ivoclar Vivadent, Inc.). One milliliter of 2-propanol (Fisher, HPLC grade) was added, and the sample was mixed by inversion six times. The sample was shaken at 120 rpm for 30 min at room temperature. The sample was briefly vortexed and then centrifuged at 10000g for 15 min at room temperature. The supernatant was transferred to a clean, dry glass test tube and evaporated under N_2

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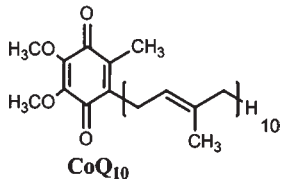

Figure 1. Chemical structure of CoQ_{10}.

gas in a Reactitherm evaporator at 40 °C. The residue was reconstituted in 1.0 mL of 2-propanol, and 350 μL of this solution was transferred to an HPLC sampling vial. HPLC-UV analysis was adapted from an analytical method for CoQ_{10} in human blood plasma developed by Mosca et al.[13] with some modifications. A volume of 40 μL of the 2-propanol CoQ_{10} extract was injected onto a Phenomenex Luna C18(2) HPLC column (4.6 × 250 mm). The isocratic HPLC mobile phase consisted of 65% ethanol/35% methanol at a flow rate of 1.0 mL/min. Total analysis time was 28 min. The HPLC system consisted of a Hitachi L-6200A Intelligent Pump with a Waters 717Plus autosampler. Detection was at 275 nm with a Waters 486 tunable absorbance detector. Data collection and analysis were performed using the Perkin-Elmer TotalChrom version 6.2 data analysis system. Linear calibration curves were produced using five concentrations of CoQ_{10} (Sigma; Avoca, Inc.) analytical standard injected in triplicate. Repeatability was assessed by measuring intra- and interday variability. Intraday variability was measured by analyzing CoQ_{10} standard solutions at two different concentrations (1.25 and 0.313 μg/mL) three times in one day, and interday variability was measured at the same concentrations over three consecutive days. The repeatability was reported as relative standard deviation (RSD, %).

Reduction of CoQ_{10} to $CoQ_{10}H_2$. CoQ_{10} most often occurs in its reduced state, $CoQ_{10}H_2$, in biological membranes. CoQ_{10} was subjected to chemical reduction with $NaBH_4$[14] to investigate the potential for our method to observe $CoQ_{10}H_2$ in the plant extracts. The experiment was performed in duplicate. CoQ_{10} standard, 2.14 mg, was dissolved in 1.0 mL of dry ethanol, and 3.0 mL of deionized H_2O was added. $NaBH_4$, 108 mg, was added to the reaction mixture and vortexed for 3 min. Reactions were incubated in the dark for 30 min. The reactions were extracted with 4.0 mL of hexane. If an emulsion formed, it was broken by the addition of 153 mg of NaCl. The hexane layer was then washed with an additional 4.0 mL of deionized H_2O. The hexane layer was next dried under a stream of N_2 gas in a Reactitherm evaporator at 40 °C. The residue was reconstituted in 1.0 mL of dry 2-propanol. Samples were analyzed by HPLC-UV as described above at 275 nm and again at 290 nm, the λ_{max} for CoQ_{10} and $CoQ_{10}H_2$, respectively. Samples were stored at 4 °C for 21 h, and the HPLC-UV analysis was repeated at both wavelengths.

Occurrence of CoQ_{10} within a Leaf. CoQ_{10} occurrence within a tobacco leaf was investigated. Three 1 cm leaf disks from the left and three analogous 1 cm leaf disks from the right of the midvein when observed from the upper surface were sampled. These leaf disks were taken within 7.6 cm of the distal end of the ninth leaf from the apex of *N. tabacum* 'KY14'. Sampling took place after plant growth for 14 weeks in the greenhouse. CoQ_{10} was extracted from each leaf disk as described above, and the CoQ_{10} contents were compared.

RESULTS

CoQ_{10} Analysis by HPLC-UV. Figure 2 shows a chromatogram resulting from the HPLC-UV analysis of a tobacco leaf. The method resulted in excellent resolution of CoQ_{10} from other lipophilic components of the 2-propanol extract with the CoQ_{10} observed at a retention time of 15.2 min. Up to three 2-propanol

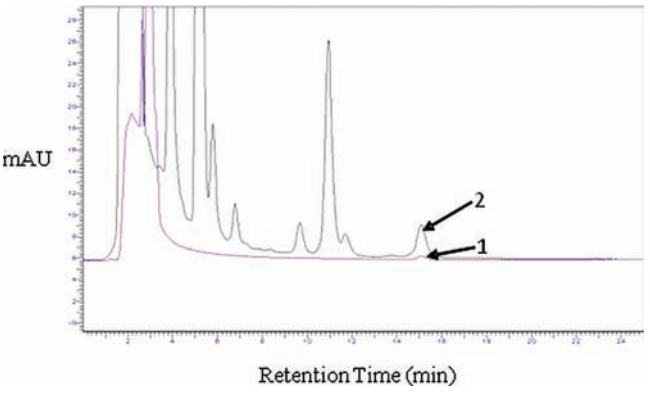

Figure 2. Typical chromatogram of CoQ_{10} standard (1.25×10^{-3} mg/mL CoQ_{10}) overlaying a typical chromatogram from tobacco extracts. 1, CoQ_{10} standard; 2, CoQ_{10} in the tobacco extract.

extractions were tested for complete extraction of CoQ_{10}, but a single extraction was found to be sufficient (data not shown).

CoQ_{10} concentrations of the 2-propanol extracts of tobacco leaves ranged from 0.40 to 10.0 μg CoQ_{10}/mL. Reproducibility of the method was assessed for injections made on the same day (intraday) and on different days (interday) and is reported as the RSD (Table 1). The intraday RSDs were 0.75 and 5.09% for 1.25 and 0.313 μg CoQ_{10}/mL, respectively. The interday RSDs were 1.10 and 4.68% for 1.25 and 0.313 μg CoQ_{10}/mL, respectively. The lower limit of detection (LoD) using 2× the standard deviation of 20 blank samples was 0.063 μg CoQ_{10}/mL.

Analyses revealed that there was no significant difference in CoQ_{10} content when sampling was done on either side of the midvein and within 7.6 cm of the leaf tip, the position specified in the sampling protocol.

CoQ_{10} and $CoQ_{10}H_2$ Qualitative Identification. The HPLC-UV method was evaluated for its ability to resolve oxidized CoQ_{10} and reduced $CoQ_{10}H_2$ and to determine that sample preparation resulted in sufficient exposure to oxygen to ensure that only the oxidized form of CoQ_{10} was recovered. Pure CoQ_{10} was subjected to chemical reduction using $NaBH_4$. The treatment resulted in total disappearance of the peak eluting at the retention time for CoQ_{10} when analyzed with detection at 275 nm and was accompanied by the appearance of a new peak at 10.0 min. When the treated samples were observed at 290 nm, the peak area for the component observed at 10.0 min increased compared to analysis of this peak at 275 nm as would be expected if that peak corresponded to $CoQ_{10}H_2$. The samples were reanalyzed by HPLC-UV at 275 nm after 21 h. Analysis showed a decrease in the peak area for the component at 10.0 min compared to the previous analysis. This was accompanied by a concurrent increase in the peak area for CoQ_{10}. This would be expected if the peak at 10.0 min represented $CoQ_{10}H_2$ becoming oxidized over this time period. This peak is absent or present only in trace amounts in CoQ_{10} extracts from tobacco following our extraction protocol, indicating that CoQ_{10} is fully oxidized, and therefore total CoQ_{10} content is represented as a result of our extraction method. $CoQ_{10}H_2$ is well resolved from other components in our analytical method, suggesting that this method could be adapted for use under properly controlled anoxic conditions to measure the oxidation state of CoQ_{10} in plant tissues.

CoQ_{10} in Leaves at Different Stalk Positions. The HPLC-UV method was used to investigate the effect of stalk position on

Table 1. Intra- and Interday Precision Analysis of the CoQ_{10} Method

linear range (μg/mL)	regression eq	corr coeff (r^2)	CoQ_{10} (μg/mL)	intraday av (μg)	RSD (%)	interday av (μg)	RSD (%)
0.158−10.14	$Y = 38778369X - 2165$	0.9992	1.25	1.15	0.75	1.16	1.1
			0.313	0.337	5.09	0.333	4.68

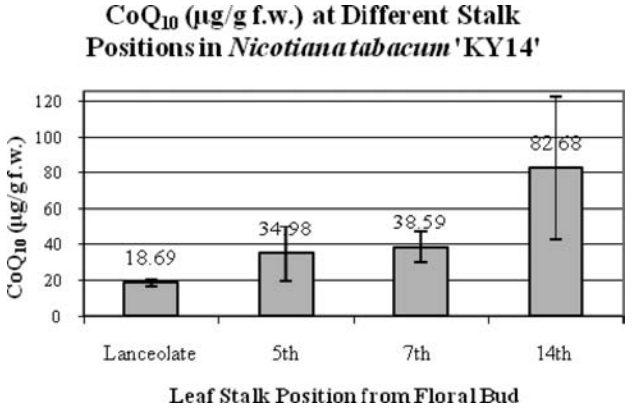

Figure 3. Average CoQ_{10} content at different stalk positions in *N. tabacum* 'KY14' (n = 5). The amount of CoQ_{10} in KY14 appears to increase as stalk position decreases from the top of the plant. The variation in CoQ_{10} content also appears to increase in this manner. Leaves were sampled after 14 weeks in the greenhouse.

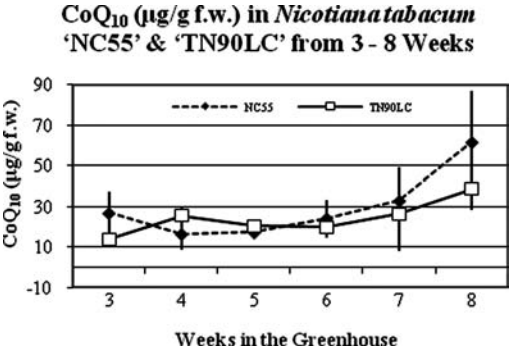

Figure 4. Average CoQ_{10} content of *N. tabacum* 'NC55' and *N. tabacum* 'TN90LC' (n = 11 for each cultivar) from 3 through 8 weeks in the greenhouse. Leaves sampled were of similar length and the fourth to fifth leaf from the apex. CoQ_{10} content in either NC55 or TN90LC appears to stay relatively the same from 4 to 6 weeks and then appears to increase through 8 weeks.

CoQ_{10} concentration in leaves of cultivated tobacco. CoQ_{10} was measured in a population of five flowering *N. tabacum* 'KY14' plants following 14 weeks of growth in the greenhouse. CoQ_{10} was extracted from one leaf disk from each plant and analyzed in triplicate. Figure 3 reveals that CoQ_{10} content increases from higher to lower leaf position down the stalk. Leaves at the midstalk positions (fifth to seventh stalk position from the floral bud) appear to have similar CoQ_{10} contents. Furthermore, variation in CoQ_{10} content increases as leaf position, length, and developmental age increases, as is indicated by the increases in standard deviation error bars. The 14th leaf is a senescing leaf.

CoQ_{10} Changes over Time. The results of our study of the correlation between whole plant age in the greenhouse and CoQ_{10} content for TN90LC and NC55 plants are summarized in Figure 4. CoQ_{10} content in NC55 appears to be relatively unchanged from 3 through 6 weeks before increasing at 7 weeks and again at 8 weeks. CoQ_{10} content in TN90LC appears to follow a similar trend, but the increase in CoQ_{10} content was of a smaller magnitude. CoQ_{10} content in both cultivars appears to remain at approximately the same level from 4 to 6 weeks of growth.

DISCUSSION

The HPLC method described here was found to be robust and reproducible when applied to the analysis of CoQ_{10} in tobacco leaves. CoQ_{10} was well resolved from other metabolites. In the current application, the method resolved CoQ_{10} from other plant isoprenoids including reduced CoQ_{10} ($CoQ_{10}H_2$), phylloquinone, and β-carotene, as well as α- and δ-tocopherols, on the basis of retention time matching with chemical standards (data not shown). The method is similar to a previous method[15] that resolves reduced CoQ_{10} from oxidized CoQ_{10} in nongreen plant tissue. The work presented here appears to be the first use for the analysis of reduced and oxidized CoQ_{10} from green tissue. The authors of the previous method cite a caution by Schindler et al.[16] that further purification is necessary to separate CoQ from other prenylquinones as their reason for avoiding green tissue; however, this did not occur with the method described here. The coelution of other isoprenoids, likely β-carotene and plastoquinone, presented a problem with the analysis of CoQ_9 from *Arabidopsis* (data not shown), indicating that our analytical method is not amenable to the analysis of CoQs that vary in prenyl side chain lengths.

Figure 3 shows the occurrence of CoQ_{10} from tobacco leaves grown in a greenhouse. The burley variety *N. tabacum* 'KY14' had increasing levels of CoQ_{10} as leaf size increased down the plant stalk from lanceolate to fully expanded leaves (leaf stalk positions 5 and 7) to senescing leaves (leaf stalk position 14). Because CoQ_{10} is an integral part of the mitochondrion, we hypothesize that the increase in CoQ_{10} content as the leaf expands may be related to an increase in mitochondrial volume or number within expanding cells, although we did not investigate this. The increase in CoQ_{10} in larger leaves may also be related to the overall age of the leaf. CoQ_{10} remained relatively stable in both NC55 and TN90LC leaves of the same size and stalk position when grown in the greenhouse from 3 to 6 weeks (Figure 4), but CoQ_{10} increased for both varieties at 8 weeks, with NC55 having the more marked response. One possibility for this again may relate to CoQ_{10} within the mitochondrion. As the leaf ages and metabolism changes with the transition to senescence, mitochondria take on a greater role in energy production while chloroplasts are dismantled.[17] For example, *Calendula officinalis* leaves continue to produce high levels of CoQ even as chloroplast-derived isoprenoids such as tocopherols and tocopherylquinones decrease.[18] Many of the chloroplast isoprenoids act as antioxidants, and as their levels decrease, so does the capacity for the plant cell to deal with oxidative stress. CoQ_{10} has been shown to increase in response to oxidative stress.[19] CoQ_{10} levels may increase within mitochondria as the leaf ages to address an increasing energy demand and/or manage oxidative stress in the decline of the influence of the chloroplast during leaf senescence.

Table 2. CoQ_{10} in Leaves of Different *Nicotiana tabacum* Varieties

tobacco variety	leaf age (weeks)	CoQ_{10} content reported reference	CoQ_{10} content in μg CoQ_{10}/g f.w.	reference	extraction; analysis
Virginia	6, green	0.016 μmol/10 g fw	1.38	20	acetone extraction followed by peroxide-free
	12, green	0.027 μmol/10 g fw	2.33		ether and alumina column chromatography
	16, dark green	0.024 μmol/10 g fw	2.07		using various concentrations of ether in
	52, yellow senescent	trace	trace		light petroleum; quantified by UV spectrometry
	52, brown	0 μmol/10 g fw	0		
White Burley	16, dark green	0.026 μmol/10 g fw	2.24		
Samsun NN[a]	not reported	7.6 ng/mg dw	0.76	13	ethanol; HPLC-UV
Petit Havana SR1	not reported	8.65 μg/g fw	8.65	22	methanol, H_2O, hexane; HPLC-UV
KY14	5, green	7.31 μg/g fw	7.31	this work	2-propanol; HPLC-UV
KY14	14, green	36.78 μg/g fw	36.78	this work	2-propanol; HPLC-UV
TN90LC	5, green	20.47 μg/g fw	20.47	this work	2-propanol; HPLC-UV
NC55	5, green	17.37 μg/g fw	17.37	this work	2-propanol; HPLC-UV
eight different varieties[b]	unknown, green	9.9−15.1 μg/g fw	9.9−15.1	21	anhydrous ethanol with sonication; LC-MS/MS

[a] By inspection of figure within reference for the wild type. [b] The eight varieties were Hong Hua Da Jin Yuan, Long Jiang 911, Yun Yan 87, Piao He 1, Yun Yan 85, K346, K326, NC89; Piao He 1 had the lowest and K326 the highest CoQ_{10} content reported.

Previous methods for measuring CoQ_{10} in tobacco leaves often featured laborious extraction steps followed by spectrometry or HPLC-UV analysis. Some of these methods are listed in Table 2 with the reported CoQ_{10} content determined when using each analytical method. CoQ_{10} content increased with leaf age from 6 to 12 weeks and decreased from 12 through 52 weeks in the Virginia cultivar studied. This supports the observed increase in CoQ_{10} reported here for the KY14 cultivar between 5 and 14 weeks. The total CoQ_{10} content reported at all leaf ages observed for the Virginia and White Burley varieties, however, is lower than the CoQ_{10} content reported for KY14, TN90LC, and NC55 at the leaf ages observed in our studies. This may be due to a difference between varieties, differences in environment, and/or possible loss of sample during the lengthy sample preparation steps used in that work.[20] The low CoQ_{10} content reported for Samsun NN may be due to a varietal difference, a difference in leaf sampling in terms of leaf stalk position and/or age, and/or a difference in sample preparation such as the use of ethanol as the extraction solvent. Growth conditions could vary enough among these separate experiments to make direct comparison difficult. The growth conditions were not reported for the Virginia and White Burley varieties, whereas the Samsun NN variety was cultivated under ambient greenhouse temperature with natural light.[12] The varieties used in our experiments were grown under ambient greenhouse temperature and natural light extended only when necessary to provide 16 h day length. A method exists for CoQ_{10} analysis in tobacco leaves by liquid chromatography coupled with mass spectrometry (LC-MS/MS) with the accompanying benefits of resolution, speed, and sensitivity (retention time of 2.93 min and LOQ of 4.0 ng/mL) that come from the use of LC-coupled mass spectrometry.[21] Although the exact growth conditions of the tobaccos used in that study were not reported, the values for CoQ are comparable to those we report for tobaccos sampled after 5 weeks in the greenhouse. In the absence of access to LC-MS/MS, the method reported here gives very good results using technology readily available to most researchers.

The results of our research have shown that temporal and spatial differences in sampling of tobacco leaves can have a significant effect on the amount of CoQ_{10} observed. For this reason, great care is required for the interpretation of previous data when they are compared to data generated in current and future studies. It is also important to carefully consider the experimental design to ensure standardization of sampling methods for CoQ. This is key to an experiment's reproducibility and is vitally important for determining real differences in CoQ_{10} content in whole plant systems. We have compiled the following suggestions for sampling of CoQ_{10} in cultivated tobacco leaves grown under conditions comparable to those described here. Leaves from plants as young as 3 weeks in a greenhouse will yield more than enough tissue. Leaf samples can be harvested from either side of the midvein within 7.6 cm (3 in.) of the leaf tip. The fifth leaf from the first leaf at the apex measuring 2.54 cm (1 in.) in length was a well-expanded leaf yielding enough tissue for analysis. Sampling from this position was high enough on the plant to avoid contact with the soil or the greenhouse floor and was easy to sample. Sampling at or near this position will also avoid the increasing variation in CoQ_{10} content we observed at leaf positions lower on the stalk. We understand these suggestions may be more or less applicable to the specific research aims of a particular project; however, they summarize the key practical application of our findings and emphasize aspects of a sampling plan that every research project of this type should address.

The method reported here provides a useful tool for the analysis of CoQ_{10} in green plant tissue that can aid in the evaluation of various conditions on CoQ_{10} accumulation, description of CoQ_{10} content in different plant species, and the screening of CoQ_{10} content in established crop species. Interest in CoQ_{10}'s myriad biological functions in plants, its possible role in plant health and resistance to environmental stresses, and the

increasing use of CoQ_{10} as a nutraceutical supplement in human health demand a variety of analytical tools necessary for its reliable analysis.

AUTHOR INFORMATION

Corresponding Author
*Phone: (919) 515-3567. Fax: (919) 515-7959. E-mail: David_Danehower@ncsu.edu.

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