Detection of carbon nanotubes in biological samples through microwave-induced heating

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ABSTRACT

We demonstrate a novel technique for quantitative detection of carbon nanotubes (CNTs) in biological samples by utilizing the thermal response of CNT under microwave irradiation. In particular, rapid heating of CNT due to microwave absorption is employed to quantify CNT uptake in agricultural samples with excellent sensitivity. We inject alfalfa (Medicago sativa) roots with a known quantity of CNT (single-walled and multi-walled) and expose the samples to a microwave field (30–50 W) to generate standard temperature–CNT concentration relationships; this detection method is then used to accurately determine CNT uptake by alfalfa plant roots grown in CNT-laden soil. The threshold for detectable CNT concentration is much lower (<0.1 μg) than common analytical methods such as electron microscopy and Raman spectroscopy. Considering the lack of effective detection methods for CNT uptake in plants, our concept is not only unique but also practical, as it addresses a major problem in the field of nanomaterial characterization and nanotoxicology risk assessment.

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1. Introduction

Carbon nanotubes (CNTs) are sp2-hybridized carbon atoms arranged in a cylindrical nanostructure. Since Iijima’s seminal 1991 paper detailing their identification [1], CNT have found a wide range of applications because of their fascinating electrical, mechanical, and thermal properties [2]. One area of potential CNT use is the area of agricultural production, such as smart delivery systems, nanoemulsions, nanosensors, and nanocatalysts for pesticides and other chemicals. Currently, thousands of chemicals are used for agricultural production throughout the United States and many of these compounds have a high adsorption affinity for organic carbon. CNT may act as a vehicle for delivery of those agricultural chemicals to the sites of toxic action or certain surfaces in pest species.1 In addition, nanomaterials may be used as modifiers of chemical behavior in the environment and in contaminant remediation that can reduce risk to non-target organisms [3,4]. CNT are not only ideal for nutrient, drug, agrochemical and biological delivery to specific cells, but also for the controlled release of these reagents as well. However, there is insufficient information and limited understanding regarding the interaction of CNT with different organisms and biological samples [5]. Data are particularly limited on the topic of CNT-plant interactions, including the potential for uptake of CNT into plants.

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To date, most studies on CNT-plant interactions have focused on the phytotoxicity (effects on germination, root elongation, plant growth, etc.) of these nanomaterials on various plant species. However, a handful of studies have reported the fate and behavior of CNT within plants. These studies demonstrate that nanotubes have the ability to cross plant cell walls and membranes which will alter essential biochemical processes necessary for plant growth and survival [6–8]. Considering the potential use of CNT in the agricultural sector and the ability of CNT to penetrate plant cells, reliable methods are critically needed to quantify CNT in plants and other biological samples in a soil environment [9]. However, existing analytical methods are limited in their usefulness in this area. The most common techniques used for the detection and characterization of CNT in such samples are scanning electron microscopy (SEM), transmission electron microscopy (TEM), and Raman spectroscopy. However, these techniques are limited in their ability to detect trace quantities of CNT within a sample.

For instance, Khodakovskaya et al. used Raman spectroscopy and TEM on tomato seeds, roots, stem and leaves in order to detect CNT uptake [6]. A very small G (Graphite) peak was observed in the magnified Raman signal, which indicates the presence of CNT inside a longitudinally cut sample of germinated tomato seed. The authors also identified CNT in TEM images, but such image analysis can be problematic and subjective. A follow-up report from the same group indicated that photothermal and photoacoustic analysis could be used to detect CNT injected directly into a plant leaf [10]. Similarly, Cañas et al. reported CNT adsorption by the plant root on the basis of SEM images of CNT on the root surface, but no CNT inside the plant root could be detected [6]. Also, near-infrared fluorescence has been used to detect the presence of CNT in macroscopic Drosophila flies whose food contained 10 ppm single-walled carbon nanotubes (SWCNT) [11]. Yang et al. utilized a similar technique to detect dye-labeled SWCNT in worms [12]. However, this technique is limited to well-dispersed-SWCNT because SWCNT do not fluoresce when they are bundled, and multi-walled carbon nanotubes (MWCNT) do not fluoresce at all. Moreover, these techniques typically require much higher concentrations than the trace quantities of interest in the case of CNT uptake by plants. Raman spectroscopy is severely limited in its ability to detect trace quantities of CNT inside a macroscopic sample. In general, SEM and TEM imaging can only be successful when CNT are visible on exterior surfaces or the surfaces of cross sections. Furthermore, all of these techniques are prone to false negatives, i.e., a lack of detection can never guarantee the absence of CNT. Finally, we note that quantitative measures of CNT concentration are difficult to establish based on these techniques.

Therefore, it is necessary to develop reliable methods to detect and quantify the presence of CNT in biological samples. CNT do have a peculiar physical property that might be leveraged to address this challenge and enable quantitative detection. Due to strong absorption of microwaves, CNT are known to evolve extreme amounts of heat when exposed to microwave field; this response is much more intense than conventional materials. This absorption also produces dramatic light emission, outgassing, and even CNT “welding” and cross-linking [13]. CNT can be heated selectively under microwave irradiation [14] to a temperature of around 2000 °C [13,15]. So far, this unique microwave-responsive property of CNT has never been used to detect CNT in plant samples and has never been utilized in any environmental application. Prior applications of microwaves to CNT include the purification and separation of different types of CNT [16,17], processing of CNT–polymer composites [18], functionalization of CNT and carbon nanohorn (CNH) by the Bingel Reaction [19–21], CNT–ceramic composite curing [22], and determination of CNT dielectric properties [15,23]. Another application of CNT-microwave heating is selective detection as well as treatment of cancer cells [24].

We hypothesized that the exposure of root samples to microwaves can not only detect the presence of CNT, but also quantitatively measure the CNT concentration in that sample. The temperature profiles and morphology would show the signatures of microwave-induced CNT heating. Thus, characterization methods based on microwave exposure may address this critical scientific challenge of CNT detection in biological samples.

2. Experimental methods

2.1. Materials

Single-walled carbon nanotubes (SWCNT) were purchased from Aldrich Chemistry. SWCNT had purity greater than 75% and diameters of 0.7–1.3 nm. Multi-walled carbon nanotubes (MWCNT) of length 10–20 μm and diameter of 30–50 nm (purity >95 wt. % and ash content <1.5 wt.%) were purchased from Cheap tubes. Sodium dodecyl benzene sulfonate (SDBS; Mw: 348.5) was obtained from Sigma Aldrich. All of these were used as received without any further purification.

2.2. Equipment

A variable power microwave generator was purchased from Optphos Instruments Inc (model number: MPG 4 RF, LAB-909) shown in Fig. S1. The generator measures incident and reflected microwave powers (0–120 W) with a resolution of 1 W. It has built-in protection to handle total reflected power. The operating frequency of the generator is 2.45 GHz. A rectangular WR-284 waveguide (able to guide 2.45 GHz frequency) was designed and built for the experiment (Fig. S2). The temperature of the sample was measured by a k-type beaded wire stainless steel thermocouple from Omega (Model SC-GG-K-30–36, ungrounded, 0.032" diameter). The thermocouple was connected to a digital multimeter (Omega model HHM290) which is able to read the temperature in the range of –200 to 1372 °C.

2.3. Preparation of CNT dispersion

Stable aqueous dispersions of CNT (SWCNT or MWCNT) were prepared using SDBS as a stabilizer [25]. SDBS (2% w/v) was completely dissolved in water by magnetic stirring and 0.2 mg/mL SWCNT (or 1 mg/mL MWCNT) were added to this solution. This mixture was then tip sonicated for 1 h at an
output power of 7 W (Misonix sonicator, XL 2000) and centrifuged (Centrifuge Centrifuge 225, Fischer Scientific) for 4 h at a speed of $\sim$5000 rpm. After centrifugation the supernatant was collected and the absorbance was measured by Shimadzu UV–vis spectrophotometer 2550 at wavelengths of 200 nm to 800 nm. Concentration of the dispersion was calculated from the absorbance (at 660 nm) using the Lambert–Beer law. The extinction co-efficient was taken as 3389 mL mg$^{-1}$ m$^{-1}$ [26]. The final concentration of the dispersion was 0.06 mg/mL for SWCNT and 0.3 mg/mL for MWCNT.

2.4. Sample preparation

2.4.1. Nanotube injected root preparation
Alfalfa (Medicago sativa) roots were taken as a representative root for method development. Alfalfa seeds were germinated and grown for 111 days in control soil (no CNT in the soil). Plant roots were collected and washed with DI water to remove soil and other particles. The primary roots (comparatively fat) were cut into small pieces. A controlled volume of CNT dispersion was injected into the root by a precision syringe (0–5 μL, Pressure–Lok Corporation, Series CG-130) to prepare the known samples for the generation of a calibration curve. The mass of CNT inside the root varied from 0 to 0.8 μg. Both SWCNT and MWCNT-injected roots were prepared to investigate the dependence on microwave heating on CNT type. A flow diagram of the overall procedure of sample preparation is shown in Fig. S3.

2.4.2. Growth of unknown sample
Dry MWCNT were mixed into soil to obtain concentrations of 1000 and 10,000 mg/kg. Alfalfa seeds were germinated and grown in the CNT-laden soil. The plant roots were collected after 14 and 111 days of growth, cleaned with DI water, immersed in a bottle containing DI water and stored in a refrigerator. These samples were used as unknown samples to determine the amount of MWCNT absorbed by the root using our calibration curve.

2.5. Experimental setup

A variable power microwave generator was connected to a microwave WR-284 waveguide. A $\sim$1 cm hole was drilled on the top of the waveguide to insert the sample holder through it. Two different power levels (30 and 50 W at 2.45 GHz frequency) were used in all experiments. The microwave generator generated the microwave power which was directed towards the sample via the waveguide. A glass sample holder was placed into the hole of the waveguide for microwave exposure. The K-type thermocouple probe was inserted into the sample to measure the temperature change. The temperature readings were obtained from a digital thermometer which was connected to the thermocouple probe. A continuous flow of nitrogen was applied directly onto the surface of the sample to prevent CNT ignition.

3. Results and discussion

The overall experimental setup to detect CNT in plant roots by microwave absorption is schematically depicted in Fig. 1. This setup bears similarity to that of Higginbotham et al. who studied the effects of CNT heating on ceramic sintering; the only difference is that there is no impedance-matched load resistor connected to the waveguide [22]. The end of the waveguide was closed with a conducting plate (a short circuit) instead of a matched load. Using a short circuit termination creates a standing wave in the waveguide because of the reflected electromagnetic waves. The electric and magnetic fields of the standing wave have maxima and minima along the waveguide. The location of the electric field maximum nearest the closed end of the waveguide is a quarter guide wavelength away (wavelength in the waveguide is different than the wavelength in air for the same frequency). This is the position where the $\sim$1 cm hole was drilled for place the sample holder. The main advantage of this setup was the magnitude of the electric field which was twice in a short circuit setup than that of the matched-load setup. As the power absorbed by the sample was proportional to the square of the electric field, this setup required lower power compared to matched-load settings.

Nanotubes display intense heating when placed in a microwave field due to strong microwave absorption [13]. The reason behind the intensive heating of CNT due to microwave irradiation is still not properly understood by the scientific community. The commonly accepted mechanism behind this response is dipolar polarization (dielectric heating) which is a strong function of the imaginary part of the dielectric constant of a material [27]. Prior studies indicate that this value is quite large (200–300) for nanotubes compared to other materials [15,23]. For this reason, nanotubes have a much more dramatic heating response to a microwave field compared to other carbon based materials. A comparison of microwave heating behavior of different sp$^2$ based carbon nanomaterials are provided in the supporting information (Table S2).

A magnified view of CNT as well as the sample heating inside the waveguide is shown in the schematic (Fig. 1). This selective heating of CNT in microwaves rapidly increases the root temperature; this temperature rise can be measured by a K-type thermocouple. The level of temperature rise depends on the CNT quantity present inside the root. This temperature rise can be plotted as a function of CNT mass to generate a calibration curve. Before generating the calibration curve, there were several variable parameters that needed to be determined: type of plant, mass of root sample, CNT mass in the sample, microwave power, and exposure time.

3.1. Microwave parameters

Alfalfa (M. sativa) seeds were germinated in control soil (no CNT present) and the roots were collected after 111 days of growth; they were kept in a refrigerator after immersion in a container full of deionized (DI) water. CNT-loaded root samples for the experiment were prepared by injecting a specified volume of CNT dispersion into small pieces of root as described in the experimental section. Root samples were dried (at $\sim$50 °C for 6 h) before microwave exposure because residual moisture can affect the actual temperature rise when the amount of CNT present in the sample is small (<1 μg). To ensure that the entire CNT sample was delivered to the sample, the volume of the dispersion was restricted to small
quantities (within 3–8 μL) such that the dispersion could not flow out of the sample. The volume of the sample was controlled simply by diluting (by adding water) or concentrating (by evaporation of water) the dispersion as required. After injection, nanotubes were trapped in the sample and could not diffuse out during drying. The sample was self-contained such that it was impossible to lose mass during the experiment except by water evaporation.

To observe the effect of plant type, two different types of root samples (alfalfa and cotton) were tested. Equal amounts (4.8 mg) of oven-dried cotton and alfalfa control roots were exposed to 30 W microwave power for 10 s. The difference between initial and final temperatures is the actual temperature rise (∆T) of the sample. For these two samples, the measured ∆T was in the same temperature range (40–43 °C), which indicated that the type of root does not strongly affect the temperature rise upon microwave exposure (Table S1). Because the microwave heating was not a function of the type of plant, the alfalfa plant root was chosen as a representative root to generate the calibration curve.

Other important parameters that were considered were the effect of sample mass and CNT mass. To test the effect of sample mass, two oven-dried alfalfa root samples (4.8 and 16 mg) were exposed to a constant microwave power (30 W). The temperature rise for the two samples versus time is shown in Fig. 2. The two curves almost overlap after 40 s of exposure. The initial discrepancy may have occurred due to the presence of a small amount of moisture. In general, this figure shows that the temperature rise due to microwave irradiation is a very weak function of root mass as long as moisture is properly eliminated. The experimental results were consistent with theoretical expectations because the samples were very small relative to the waveguide dimensions (16 × 4 cm). Moreover, the electromagnetic field was also uniform at the position where the sample was placed. Therefore, variations in mass for such relatively small samples should not affect the temperature rise.

The experimental results indicate that temperature rise (∆T) depends strongly on microwave power and exposure time, and it does not depend on the type and mass of root samples. As long as microwave power and exposure time remain constant, ∆T will be a function of CNT present inside the root sample. Thus, this technique does not require normalization of CNT mass with the root sample mass. The critical parameter for the calibration curve is CNT mass injected into the sample. The mass of roots in the calibration samples varied between 4 and 18 mg and the amount of CNT injected was in the range of 0–0.8 μg.

### 3.2. Generation of calibration curves

Before generating the calibration curve, several baseline samples were tested for a given microwave power. These baseline trends included the thermocouple itself, a control root, a sodium dodecyl benzene sulfonate (SDBS) injected sample and single-walled carbon nanotube (SWCNT) injected samples. Fig. 3 demonstrates the temperature profile for the thermocouple, control sample, SDBS-injected sample, and different SWCNT-loaded samples (0.18 and 0.78 μg SWCNT) upon prolonged exposure to 30 W applied microwave power. Although a rise in temperature was observed for the thermocouple and for the control root, the heating was not significant compared to that observed for the SWCNT-loaded sample. The thermocouple heating was minimal (maximum of ~50 °C for 2 min of exposure) compared to the control (~95 °C for 2 min exposure) and nanotube injected roots. After an initial rapid rise in temperature, the thermocouple and control root reached a steady state. For the 0.18 μg SWCNT-loaded sample, the

Fig. 1 – A schematic view of the experimental setup. A magnified view of the root shows elevated temperatures inside the root due to CNT heating during microwave exposure.
temperature steadily rose to 237 °C after 2 min of exposure and then started to decrease; this might have occurred due to gradual SWCNT oxidation. Note that long (>2 min) microwave exposure times may degrade SWCNT in contrast to repeated short exposure times (6–10 s). In contrast, the root sample with 0.78 µg SWCNT reached ~500 °C within only 40 s of exposure, indicating the ignition of SWCNT despite the nitrogen blanket. Even if there is a continuous flow of nitrogen, it is almost impossible to achieve a 100% inert atmosphere inside the sample vial. As a result, the SWCNT sample is likely to be oxidized when the temperature reaches ~300 °C. Similar behavior was observed for MWCNT as well.

In order to quantify the heating behavior of the CNT stabilizer (SDBS) in a microwave field, 0.26 mg of SDBS-injected root was tested at the same microwave power (30 W); this was the amount of SDBS present in the root when 0.78 µg SWCNT was injected. The SDBS-injected root sample displayed some increase in temperature rise relative to the
control root sample; again, it was not comparable to SWCNT-injected samples. These results indicate that the SWCNT-free samples have negligible effects on temperature rise, in comparison with the effects of the SWCNT-loaded samples.

Calibration curves were generated at 30 W and 50 W microwave power for 10 and 6 s, respectively. Both SWCNT and MWCNT were evaluated in this study. Figs. 4 and 5 depict calibration curves at different powers generated for different types of CNT. CNT-injected root samples (mass of samples varied from 4–18 mg) were exposed to the microwave and the temperature rise ($\Delta T$) was recorded and plotted as a function of CNT mass (0–0.8 μg). These figures showed that the measured value of $\Delta T$ was higher with the increase of microwave power even though the exposure time to the microwave was less. Again, the most important feature of the calibration curves was the detectable limit of CNT (≤0.1 μg) as shown in Figs. 4 and 5; this value is far lower than any other competing methods (SEM, TEM or Raman spectroscopy). The samples were also examined at lower microwave power (15 W) for 20 s. At this power level, the threshold for detecting CNT becomes higher as the difference of temperature rise between control sample and smaller CNT loaded samples (0.1–0.18 μg) cannot be distinguished clearly. Since nanotubes are more sensitive to higher power (30–50 W) even for short time of exposure (~10 s), the detectable threshold can be moved lower simply by raising the microwave power or exposure time associated with a given calibration curve.

The effect of differing CNT types (SWCNT vs. MWCNT) is also illustrated in Figs. 4 and 5. According to the calibration curves (30 and 50 W), $\Delta T$ was slightly lower for MWCNT-injected samples than SWCNT-loaded samples. This is in agreement with the study by Higginbotham et al. who showed that the initial heating response of SWCNT and MWCNT loaded ceramic samples were almost the same when exposed to microwaves [24].

To check the repeatability of the method, two sets of samples were prepared and tested at two different microwave powers (30 and 50 W). Both of these independent measurements displayed similar trends of the temperature–CNT concentration relationship. Moreover, the samples were tested at least 10–15 times each and the results were replicable within a certain range of temperatures. For temperatures below ~250 °C, the method was nondestructive and the samples repeatedly displayed the same heating behavior, indicating that the roots are merely heated and not degraded. In the calibration curves shown in Figs. 4 and 5, the maximum temperatures found for each sample were considered because this was the most accurate measurement of the true temperature rise. There are a number of factors, including thermocouple placement relative to the root that could yield artificially low values for $\Delta T$. It is also possible that CNT loading (and thus CNT-induced heating) in the root is non-uniform. This non-uniform loading creates non-homogeneous heating of the sample, so maximum readings are the appropriate measure for the calibration curves. The average and standard deviation for $\Delta T$ measurements were also recorded and plotted in Figs. S5–S8.

3.3. Method confirmation

To test the reliability and accuracy of our microwave-assisted detection technique, we carried out a set of proof-of-concept studies. A blind analysis was conducted with seven SWCNT-injected root samples. All the root samples were exposed to 30 W (for 10 s) and 50 W (for 6 s) microwave power; $\Delta T$ measurements were recorded and then analyzed using the calibration curves. Table 1 shows the readings from the calibration curves and also the actual SWCNT injected into the sample. The experimental results suggest that for samples with more than 0.1 μg SWCNT present, two independent

Fig. 4 – Calibration curve generated by using known samples injected with known quantities of CNT (SWCNT and MWCNT) at 30 W microwave power for 10 s exposure. The root mass of the SWCNT-injected samples varied from 12 to 18 mg, and MWCNT-injected root mass varied from 4–11 mg. The samples were tested at least 10–15 times each.
measurements yielded very similar and almost accurate estimates for the true CNT loading. The uncertainty rises below this threshold. This problem can easily be avoided by using a calibration curve at a higher microwave power or for a longer time at a lower microwave power. These results demonstrate the utility and versatility of the method. Additionally, the microwave detection technique was justified by thermogravimetric analysis (TGA) [28]. The details of this analysis is described in the supporting information. Also, SEM was conducted on the cross section of the root sample; as expected, this type of detection yields uncertain results, in contrast to the microwave-based method (Fig. S9) [6].

3.4. Quantitative detection of CNT

After establishing the reliability of the method, we conducted quantitative detection of CNT on several root samples with unknown CNT loading grown in different CNT-laden soils. Alfalfa roots were collected from the plants that have been grown in MWCNT laden soil (1000 and 10,000 mg/kg) and taken after 14 and 111 days of growth. The unknown samples were named as sample A: alfalfa plant grown for 14 days (1000 mg/kg MWCNT in soil), sample B: alfalfa plant grown for 111 days (1000 mg/kg MWCNT in soil), and sample C: alfalfa plant grown for 14 days (10,000 mg/kg MWCNT in soil). Raman spectroscopy was performed on these samples to detect the presence of MWCNT. However, Raman spectra showed no indications of CNT in any of the above samples; of course, such measurements can easily give a false negative, so the presence of CNT remains unknown. In contrast, our microwave method showed that sample B adsorbed 0.1 μg (using the 30 W calibration curve) or 0.09 μg (using the 50 W calibration curve) MWCNT in the root; the presence of nanotube in this sample was qualitatively confirmed by TGA (Fig. S10).

Fig. 5 – Calibration curve generated by using root samples injected with known quantities of CNT (SWCNT and MWCNT) at 50 W microwave power for 6 s of exposure. The samples were tested at least 10–15 times each.

Table 1 – Blind test analysis of SWCNT-loaded samples.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Mass of root, mg</th>
<th>Power, W</th>
<th>Time, s</th>
<th>ΔT, °C</th>
<th>Mass of SWCNT, μg (from calibration curve)</th>
<th>Actual SWCNT present, μg</th>
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<tr>
<td>1</td>
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<td>30</td>
<td>10</td>
<td>70</td>
<td>0.10</td>
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<td>50</td>
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<tr>
<td>2</td>
<td>1.6</td>
<td>30</td>
<td>10</td>
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<td></td>
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<td>50</td>
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<td>3</td>
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<td></td>
<td>50</td>
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<td>67</td>
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<tr>
<td>4</td>
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<td>10</td>
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However, the $\Delta T$ values for samples A and C indicated the absence of MWCNT according to both calibration curves. This agreement between two individual measurements confirms the reliability of the reading and also confirms that the microwave technique is significantly more sensitive than alternative techniques. The test results using different calibration curves (for different microwave power levels) are presented in Table 2.

In future studies, we plan to use this method to explore the effects of MWCNT on the germination, short and long term growth, water use efficiency and photosynthetic rate on different plant species (alfalfa, corn, cotton, and sorghum). Preliminary data (Table 2) indicate that the microwave heating method provides a means to quantify CNT which may be useful in explaining any observed toxicity with the actual amount of nanotubes adsorbed by the plant.

4. Conclusion

The use of microwave-heating for CNT detection in environmental samples is entirely novel and addresses a wide-ranging obstacle in the field of nanotoxicology. Given the potential for CNT to be used in agricultural and environmental applications, it is important that the effects of interactions of CNT with crop species be evaluated. The use of this method is thus invaluable to effectively quantify CNT in crop species. The versatility of this technique is not limited to agricultural samples; this is also applicable to other biological and environmental samples. In addition, this method can easily be developed due to its low cost, repeatability and reliability of detection. Our future studies will investigate the detection of CNT in soil, earthworms, and tissue samples. Data from these studies will be useful to a wide range of scientists, from environmental chemists or engineers to government scientists who create or utilize nanomaterials and are charged with ensuring the safety of nanomaterials.

5. Supporting information

Figures depicting microwave waveguide and generator, experimental procedure, effect of type of samples, issues with thermocouple placement, standard deviation plots, SEM, and TGA analysis are available in the supporting document.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbon.2012.05.022.

REFERENCES


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### Table 2 – Detection of MWCNT in unknown sample.

<table>
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<th>Sample</th>
<th>Mass of root, mg</th>
<th>Power, W</th>
<th>Time, s</th>
<th>$\Delta T$, °C</th>
<th>Mass of MWCNT, µg (from calibration curve)</th>
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