ELECTROCHEMISTRY

Determination of Malachite Green in Aquaculture Water by Adsorptive Stripping Voltammetry

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ABSTRACT
An adsorptive stripping voltammetric method for the determination of malachite green in aquaculture water has been developed. Initial studies were made using the cyclic voltammetry of malachite green at a glassy carbon electrode in 0.1 M phosphate buffer from pH 2 to 10. The redox behavior observed for malachite green was verified by the characterization of malachite green and its reduction product, leucomalachite green. Furthermore, leucomalachite green was found not to interfere with the determination of malachite green at pH 7.4, the optimum pH for malachite green determination. As a result, further studies were performed using adsorptive stripping voltammetry for the determination of malachite green in aquaculture water. The voltammetric waveform, accumulation potential, and accumulation time were optimized. The calibration plot was linear from 0.2 µM to 1.2 µM for malachite green using differential pulse voltammetry with a sensitivity of 0.8311 µA/µM. Using the method of multiple standard addition, aquaculture water fortified with 0.5 µM and 0.75 µM malachite green provided mean recoveries of 78.79\% and 87.20\% with coefficients of variation of 2.07\% and 1.45\%. Therefore, analytical figures of merit suggest that this method provides rapid, simple, economical, and precise determination of malachite green in aquaculture water.

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Introduction
Malachite green (C\textsubscript{23}H\textsubscript{25}N\textsubscript{2}Cl) is a member of the triphenylmethane class of dyes and is known to exhibit both antimicrobial and antiparasitic properties. It has been extensively used as a biocide in the aquaculture industry worldwide (Aiderman 1985). However, malachite green and its reduced form, leucomalachite green, have been shown to have toxic effects on the human immune system, reproductive system, and to be potential carcinogens (Meyer and Jorgenson 1983; Gouranchat 2000; Srivastava, Sinha, and Roy 2004; Sudova et al. 2007). Although malachite green is not approved for use in aquaculture in Canada, the United States (Hernando et al. 2006), Europe (Commission of the European Communities 2002), and Japan, its illegal use will probably continue due to its low cost, wide availability,
and effectiveness as a pesticide. In addition to its use in aquaculture, malachite green has been widely used as a dye in the textile industry, as well as a food additive and coloring agent (Culp and Beland 1996). A report prepared by the Water Research Centre for the Department of the Environment, Transport, and the Regions of the United Kingdom recommended an annual average environmental quality standard of 500 ng/L malachite green for the protection of freshwater aquatic life (Burchmore and Wilkinson 1993). Consequently, in order to preserve the environment and protect human health, it is important to develop analytical methods for malachite green.

A number of techniques have been developed for determination of malachite green, including high performance liquid chromatography (HPLC) (Swarbrick, Murby, and Hume 1997; Bergwerff and Scherpenisse 2003; Mitrowska, Posyniak, and Zmudzki 2005; Bajc, Doganoc, and Šinigoj Gačnik 2007; Long et al. 2008; Xie et al. 2013; Furusawa 2014) and liquid chromatography-tandem mass spectrometry (Hidayah et al. 2013; Lopez-Gutierrez et al. 2013; Nebot et al. 2013). These methods provide high sensitivity and excellent accuracy. However, these rely on relatively expensive instruments that need to be operated by well-trained personnel. In addition, the sample preparation using these methods may be complex and time-consuming.

Electrochemical sensors have been widely used for the determination of contaminants in the environment and food industries (Privett, Ho Shin, and Schoenfisch 2010) due to high sensitivity, good selectivity, rapid response, low cost, and the possibility for use in the field. The electrochemical determination of malachite green at glassy carbon electrodes modified with carbon nanotubes or colloidal gold-chitosan carbon nanotubes has been reported (Yi, Qu, and Huang 2008; Y. Y. Liu et al. 2014). The addition of an anionic surfactant to the sample to enhance the signal has also been investigated (Huang et al. 2008). A modified carbon paste electrode based on a self-assembly layer of ethylenediamine and graphene oxide has been recently reported (Zhang et al. 2012).

To the best of our knowledge, the electrochemical properties of malachite green and the mechanisms underlying its voltammetric behavior have not been studied in-depth. Therefore, to address this, the main objectives of this current work are to study the electrochemical properties of malachite green at an unmodified glassy carbon electrode and to develop a convenient electrochemical method for its determination in water.

In the present investigation, the electrochemical behavior of malachite green was investigated over a wide pH range by cyclic voltammetry. The effect of scan rate was examined at pH values between 2.0 and 10.0 and possible mechanisms for its voltammetric behavior proposed. In addition, further cyclic voltammetric investigations were made on leucomalachite green, the reduction product of malachite green, to further elucidate the redox mechanism of malachite green. Studies were then conducted to explore the possibility of exploiting these findings to develop a method for the adsorptive stripping voltammetric determination in aquaculture water at an unmodified glassy carbon electrode by optimizing the accumulation potential, accumulation time, and electrochemical measurement waveform. This paper presents a detailed description of these studies.

**Experimental**

**Apparatus and chemicals**

Cyclic voltammetry was performed using an Autolab potentiostat interfaced to a PC for data acquisition and instrument control via the Nova operating system (Autolab, The
Netherlands). The voltammetric cell contained a graphite rod counter electrode, a silver/silver chloride reference electrode (Ag/AgCl, with 3 M KCl solution) (Chen Hua Instruments, Shanghai, China), and a 3 mm diameter glassy carbon electrode as the working electrode (Chen Hua Instruments, Shanghai, China). Cyclic voltammograms were initially recorded in 0.1 M phosphate buffer, then in the buffer containing malachite green. For initial studies, a starting potential of 0.0 V with a switching potential of +1.0 V was employed. Differential pulse voltammetry was performed using a starting potential of 0.0 V and a final potential of +1.0 V with a step height of 10 mV, pulse repetition time of 0.2 s, pulse amplitude of 100 mV, and pulse duration of 50 ms.

All chemicals were supplied by Fisher Scientific (China), unless stated otherwise. Malachite green was obtained from Tanmo Quality Inspection, Beijing, China. Leucomalachite green was obtained from Dr. Ehrensorfer, Germany and was analytical grade. A 10 mM malachite green stock solution was prepared by dissolving the appropriate mass in ultrapure water. Working standards were prepared by dilution of the stock solution with ultrapure water. Ultrapure water was obtained from a Milli-Q Academic System (Millipore, USA). Solutions of disodium, trisodium, sodium o-phosphate, and o-phosphoric acid (Sinopharm Chemical Reagent Company, China) were made at a concentration of 0.2 M by dissolving the appropriate mass in ultrapure water. These were then combined to provide the desired pH in 0.9% NaCl. An appropriate volume was then added directly to the voltammetric cell and diluted with sufficient ultrapure water to give an overall phosphate concentration of 0.1 M.

**Water sample collection and pretreatment**

The water sample was obtained from Badu River Channel, Jumahe River Basin, Fangshan District, Beijing, 14 August, 2014 at a depth of 0.5 m. A 2.5 L glass hydrophore was used to collect the water which was transferred directly into sample bottles. Samples were refrigerated and sent to the laboratory as soon as possible for analysis. These were then passed through a microporous membrane filter (water-system, 0.22 µm) prior to voltammetric analysis.

**Results and discussion**

Figures 1–4 show the cyclic voltammograms obtained for a 1.0 mM solution of malachite green in 0.1 M pH phosphate buffer at pH values 2.0, 4.0, 7.4, and 10 at scan rates between 20 mV/s and 200 mV/s. Previous reports have shown that at pH 2.0 the electrochemical oxidation of malachite green leads to the formation of the oxidized form of \( N,N,N,N \)-tetramethylbenzidine (\( [1,1\text{-biphenyl}-4,4\text{-diamine}] \)) characterized by quasi-reversible diffusion controlled behavior (Ngamukot et al. 2006; Ma et al. 2008) \( 2e^-, 2H^+ \) redox couple (Galus and Adams 1962). In the present study, the cyclic voltammograms at pH 2.0 (Figure 1) showed similar behavior, with a single oxidation peak on the initial positive scan and single reduction peak on the return negative scan. Plots of peak current (\( i_p \)) vs. the square root of scan rate (\( v^{1/2} \)) showed a linear dependence, showing the electrochemical process to be diffusion controlled. Consequently, malachite green provided similar redox behavior at this pH to the results provided in the literature.

Figure 2 shows a cyclic voltammogram obtained at pH 4.0. The oxidation at pH 2.0 is still present, but the reduction peak on the return negative going scan is much broader and
less well-defined. Several other redox processes were also present because the pH was close to the pKₐ value of malachite green. These conditions result in two or more forms of malachite green being present producing the additional peaks. However, at pH 7.4 (Figure 3) and at values between 6.0 to 8.0 along with this same oxidation peak at +0.90 V, an additional oxidation peak is seen (Eₚ = +0.5 V). The peak current values for this oxidation peak were linearly related to scan rate (ν). Further investigations of current function (iₚ/ν¹⁄₂) vs. the square root of scan rate (ν¹⁄₂) (Nicholson and Shain 1964; Wopschall and Sharin 1967) demonstrated this process to be reactant adsorption in nature. On the return negative scan, a single reduction peak was present at +0.4 V. In addition, there was little

Figure 1. Cyclic voltammograms of 1.0 mM malachite green in 0.1 M phosphate buffer at pH 2.0 at scan rates of (a) 20, (b) 50, (c) 100, (d) 150, and (e) 200 mV/s.

Figure 2. Cyclic voltammograms of 1.0 mM malachite green in 0.1 M phosphate buffer at pH 4.0 at scan rates of (a) 20, (b) 50, (c) 100, (d) 150, and (e) 200 mV/s.
response for leucomalachite green by cyclic voltammetry at pH 7.4. Thus, leucomalachite green would not interfere with the determination of malachite green at this pH value.

It is believed that the oxidation peak at $+0.5\, \text{V}$ resulted from the oxidation of the carbinol form of malachite green. At pH values higher than 6.9 (Cuong, Ishizaka, and Kitamura 2012), malachite green was chemically reduced to its carbinol form, which may undergo a two electron oxidation to provide malachite green and hydroxyl ion. The carbinol form is more nonpolar and hence would explain the reactant adsorptive behavior observed. The more positive oxidation at $+0.90\, \text{V}$ is believed to result from oxidation of the amine nitrogen lone electron pair to form a radical species, as shown in Equation (1). This
phenomenon has also been reported previously (Masui, Sayo, and Tsuda 1968).

\[ R_3 - N_2^* \rightarrow R_3 - N_2^+ + e^- \]  \hspace{1cm} (1)

Figure 4 shows that at pH 10, one oxidation peak was recorded across the potential range that was caused by the oxidation of the amine. The high pH and high concentration of OH\(^-\) makes the oxidation to be unfavorable. In some scans at this pH, an additional oxidation peak was observed that was attributed to the instability of malachite green at this high pH.

To further investigate the electrochemical mechanism for malachite green, the cyclic voltammetry of leucomalachite green was also investigated under the same conditions. The same oxidation peak for malachite green was also recorded for leucomalachite green (Figure 5). Moreover, the solution around the working electrode was seen to change from clear to green when the potential was scanned from 0.0 V to +0.8 V at pH 2.0. It was concluded that this green color resulted from electrochemically generated malachite green formed by the oxidation of leucomalachite green to malachite green. This phenomenon was not observed when the supporting electrolyte pH was increased. Further cyclic voltammetric investigation of leucomalachite green showed a second peak that became larger as the pH was changed to 4.0. However, the peak potential remained constant (Figure 6). This result may be due to the oxidation of the carbinol form of leucomalachite green as supported by the results. The voltammetric peaks of leucomalachite green decreased in magnitude with increasing pH, as it was more difficult to oxidize to malachite green under alkaline conditions.

Additional studies were made into the origin of the reduction peak recorded at +0.4 V for malachite green. A series of cyclic voltammetric investigations were undertaken using a switching potential of +0.6 V, before the beginning of the second oxidation. Consequently, it was concluded that this reduction was the result of the species formed from the oxidation at +0.5 V. An additional two oxidation peaks were found when extending the switching

Figure 5. Cyclic voltammograms of (a) 1.0 mM malachite green and (b) leucomalachite green in 0.1 M phosphate buffer at pH 2.0.
potential to the more positive potential of $+2.0 \text{ V}$, which was believed to result from the polymerization of malachite green (De-Lin et al. 1989; Raoof, Ojani, and Baghayeri 2013) at its amine group (Chen, Chen, and Thangamuthu 2007). The mechanism of the voltammetric dimerization of aromatic amines has been investigated by Hart, Smyth, and Smyth (1981). Several mechanisms for the polymerization of malachite green have been proposed (De-Lin et al. 1989; Raoof, Ojani, and Baghayeri 2013). However, we believe that the tail-to-tail dimerization (Hart, Smyth, and Smyth 1981; Honeychurch, Hart, and Kirsch 2004) shown in Scheme 1 explains the voltammetric behavior observed in this study. Malachite green (i) was first oxidized to a cation radical (ii). This radical (ii) may form the resonance structure (iii). The presence of (iii) may cause the electrophilic tail-to-tail dimerization of two radical species to provide the dimer (iv) that may be converted to (v) by a two electron, two hydrogen ion oxidation.

The reactant adsorption of the malachite green at neutral pH values is analytically a very useful finding as it allows for the development of an adsorptive stripping voltammetric method for the determination of malachite green. Consequently, this approach was investigated in detail.

**Adsorptive stripping voltammetry**

**Effect of accumulation potential**

Figure 7 shows the effect of accumulation potential on the resulting peak current of both oxidation peaks using an accumulation time of 15 s. The peak current increased from $+0.6 \text{ V}$ to a maximum value between $+0.4 \text{ V}$ and $-0.2 \text{ V}$ vs. Ag/AgCl which was found to decrease at more negative potentials. Consequently, further studies were carried out using an accumulation potential of $0.0 \text{ V}$ vs. Ag/AgCl. When using longer accumulation times or more negative accumulation potentials, two oxidation peaks were recorded at peak values of $+0.54 \text{ V}$ and $+0.63 \text{ V}$. The more positive peak is believed to result from the oxidation of a monolayer of malachite green on the glassy carbon electrode surface, whereas the more
negative peak results from the oxidation of a multilayer of malachite green deposited on the glassy carbon electrode. This more negative peak was only seen at extended accumulation times, as it was only formed once the monolayer had been established (Honeychurch, Hart, and Cowell 2000).

**Effect of accumulation time**

Figure 8 shows the effect of increasing accumulation time at an applied potential of 0.0 V using 2.0 µM malachite green. The first oxidation stripping peak was found to increase with

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**Scheme 1.** The electrochemical polymerization of malachite green.

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**Figure 7.** Effect of accumulation potential for 10 µM malachite green at pH 7.4, 0.1 M phosphate buffer.
increased accumulation time and reached a maximum value at ca. 100 s. The second oxidation peak was also found to increase with accumulation time over the time frame studied.

**Effect of electrochemical measurement waveform**

In order to improve the detection limit, differential pulse voltammetry was investigated for the determination of malachite green. Differential pulse voltammetry provides improved signal-to-noise ratios and low limits of detection (Honeychurch, Hart, and Cowell 2000). Initial studies were performed under the optimized conditions by differential pulse voltammetry. The resulting oxidation peak of malachite green was greatly improved and additional smaller peaks were also observed (Figure 9). Consequently, differential pulse voltammetry was used for subsequent investigation.

**Analytical figures of merit**

A calibration study was carried out using differential pulse voltammetry in a 0.1 M pH 7.4 phosphate buffer from 0.2 µM to 2 µM. The plot was linear up to 1.2 µM with a slope of 0.8311 µA/µM and an $R^2$ value of 0.990. The limit of detection based on a signal-to-noise ratio of 3 was 0.12 µM. Beyond 1.2 µM, the response was found to be quasi-linear up to at least 2 µM. These performance characteristics are able to meet the detection limits required for the determination of malachite green in environmental water samples (Burchmore and Wilkinson 1993). It should be mentioned that by simple extension of the relatively short accumulation times investigated in this study it would be possible to improve these analytical figures of merit. Table 1 provides a summary of previously reported methods for the determination of malachite green in various water samples. The spectrographic and chromatographic approaches require a separate concentration and clean-up step to obtain...
the reported detection limits. In this method, the preconcentration and measurement are performed in the same solution, eliminating problems such as losses in sample preparation. This approach uses an unmodified carbon electrode that has the added advantage of being more stable compared to techniques based on modified electrodes.

**Interference studies**

As mentioned previously, cyclic voltammetric investigations showed that under the optimized conditions, equal molar concentrations of leucomalachite green did not interfere with the determination of malachite green. To further validate the performance of the method, a number of possible interferences were studied. Thirteen metal and acidic ions previously reported to be common interferences were evaluated (Lin et al. 2013; Y. Sun et al. 2015) for the determination of 1 µM malachite green. No interferences were observed for greater than 500-fold excess concentrations of $K^+$, $Na^+$, $Ca^{2+}$, $Fe^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Cl^-$, $I^-$, $SO_4^{2-}$, $NO_3^-$, $CO_3^{2-}$, $PO_4^{3-}$, and $CH_3COO^-$.

**Analysis of aquaculture water**

The analytical procedure was evaluated by the determination of malachite green in aquaculture water with and without fortification of malachite green standards. The samples were diluted one-to-one in 0.2 M pH 7.4 phosphate buffer, and the concentration of malachite green was determined using the method of standard addition. Table 2 shows the precision and recovery data obtained for replicate analysis of a single aquaculture water sample fortified with 0.50 µM and 0.75 µM malachite green. These results demonstrate that this method has promise for the determination of malachite green in water samples. Further analysis of other environmental water samples showed similar good recoveries and precision.
| Technique                        | Linear dynamic range | Detection limit | Sample                  | Comments                                                                                                                                  | Reference                      |
|---------------------------------|----------------------|-----------------|-------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|
| Spectrophotometric              | 50–250 µg/L          | 2.2 µg/L        | River water             | Diffuse reflectance spectrophotometry 635 nm using sodium dodecyl sulfate as a counter ion.                                               | Robaina, dos Reis, and Cassella (2011) |
| Spectrophotometric              | 3.5 µg/L to 183 µg/L | 1.5 µg/L        | Tap water               | Cloud point extraction procedure using pH-sensitive hydrogel. Measurement at 617 nm.                                                   | Bahram, Keshvari, and Najafi-Moghaddam (2011) |
| Spectrophotometric              | Malachite green, 9.9 µg/L to 800 µg/L; crystal violet 16 µg/L to 1000 µg/L | Malachite green 2.9 µg/L; crystal violet 4.8 µg/L | Pool and river water | Spectrophotometric determination of malachite green and crystal violet at 624 and 579 nm, respectively, following cloud point extraction. | An et al. (2010) |
| Spectrophotometric              | 0.50 µg/L to 250 µg/L | 0.28 µg/L       | Fish farm water         | Malachite green and leucomalachite green isolated with maghemite nanoparticles modified with sodium dodecyl sulfate followed by magnetic separation. | Afkhami, Moosavi, and Madrakian (2010) |
| Spectrophotometric              | 2.9 µg/L to 73 µg/L  | 0.73 µg/L       | Surface water           | Method is based on the catalytic effect of silver nanoparticles on the oxidation of malachite green by hexacyanoferrate (III) in acetate-acetic acid medium measured at 610 nm. | Sahraei et al. (2013) |
| Spectrophotometric              | 3.2 µg/L             |                 | Water                   | Malachite green extracted with sodium dodecyl sulfate coated alumina solid phase. Ultraviolet detection at 617 nm.                  | Farhadi et al. (2010)            |
| Spectrophotometric              | 0.5 to 1.0 mg/L      | –               | Drinking and river water| Malachite green preconcentrated from 1 L of water with magnetic solid phase extraction using magnetic affinity adsorbent                  | Šafařka and Šafaříková (2002)   |
| High-performance liquid chromatography | 0.20 to 100 µg/L   | 0.01 µg/L       | Yangtze River and pond waters | HPLC-Ultraviolet detection at 600 nm, following three-phase hollow fiber liquid phase microextraction | Zou et al. (2014)               |
| Method                                                                 | Detection Range          | Detection Limit       | Sample Type          | Description                                                                                           | Reference                           |
|-----------------------------------------------------------------------|--------------------------|-----------------------|----------------------|-------------------------------------------------------------------------------------------------------|-------------------------------------|
| High-performance liquid chromatography                                  | 0.2 to 100 µg/L          | 0.1 µg/L              | Fish farm water      | Dispersive liquid–liquid microextraction. Molten polymer solid phase extraction of malachite green followed by molecularly imprinted polymer solid phase extraction. | Maleki, Farhadi, and Nikkhahi (2012) |
| High-performance liquid chromatography                                  | 0 to 200 µg/L            | 0.05 µg/kg; seawater 0.1 µg/L | Seafood, seawater    | Molecularly imprinted polymer solid phase extraction of malachite green followed by molecularly imprinted polymer solid phase extraction. | Lian and Wang (2012)               |
| High-performance liquid chromatography                                  | 0.02–10, 0.5–100 and 0.2–100 µg/mL for MG, enrofloxacin and ciprofloxacin, respectively. | 0.01, 0.07 and 0.10 µg/L for MG, enrofloxacin and ciprofloxacin, respectively. | Fish farm water | Solid phase extraction of malachite green, enrofloxacin, and ciprofloxacin | H. W. Sun et al. (2011)            |
| High-performance liquid chromatography                                  |                         |                       | Fish and river water | Solid phase extraction using a column with a monolithic molecularly imprinted epoxy resin-based polymeric stationary phase. | Wang et al. (2009)                 |
| High-performance liquid chromatography with tandem mass spectrometry   |                          | 1.5 ng/L              | Water samples from rural Northwestern Spain | Solid phase extraction | Iglesias et al. (2014) |
| Micellar electrokinetic chromatography                                 | 0.1 to 10 µg/L           | 69.6 pg/mL            | Aquaculture water    | Cloud point extraction followed by micellar electrokinetic chromatography | Luo et al. (2010)                  |
| Electrochemical detection                                              | 18.4 µg/L to 82.2 mg/L   | 2.2 µg/L              | Water samples        | Glassy carbon electrode modified with multiwalled carbon nanotubes. | Yi et al. (2008)                   |
| Electrochemical detection                                              | 0.35 µg/L to 1.83 mg/L   | 0.33 µg/L             | Pond water           | Glassy carbon electrode modified with multiwalled carbon nanotubes. Quantification cyclic voltammetry. | L. Q. Liu et al. (2009)            |
| Adsorptive stripping voltammetry                                       | 43.79 µg/L to 437.9 µg/L | 43.79 µg/L            | Aquaculture water    | Dilution with phosphate buffer. Unmodified glassy carbon electrode | Present study                      |
Conclusions

The redox behavior of malachite green was investigated at a glassy carbon electrode and found that well-defined peaks were obtained in 0.1 M pH 7.4 phosphate buffer using cyclic voltammetry and differential pulse voltammetry. This is the first report to investigate the voltammetric behavior of malachite green over an extended pH range. It is also the first report to exploit the oxidation process seen at pH 7.4 at an unmodified glassy carbon electrode for the determination of malachite green in aquaculture water. Unlike previously reported methods, it was shown that no elaborate extraction or separation procedures were required as the method of multiple standard additions was shown to be both precise and accurate (Table 2). It should be possible to improve the performance characteristics of the method by the application of longer accumulation times.

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Table 2. Recovery and precision for malachite green in aquaculture water.

| Sample | Original concentration (µM) | Added (µM) | Found (µM) | Recovery (%) |
|--------|-----------------------------|------------|------------|--------------|
| 1      | not detected                | 0.50       | 0.406      | 81.24 ± 0.36 |
| 2      | not detected                | 0.50       | 0.387      | 77.42 ± 0.20 |
| 3      | not detected                | 0.50       | 0.401      | 80.22 ± 0.18 |
| 4      | not detected                | 0.50       | 0.389      | 77.86 ± 0.32 |
| 5      | not detected                | 0.50       | 0.386      | 77.20 ± 0.27 |
| 1      | not detected                | 0.75       | 0.660      | 89.20 ± 0.28 |
| 2      | not detected                | 0.75       | 0.663      | 88.80 ± 0.32 |
| 3      | not detected                | 0.75       | 0.658      | 87.87 ± 0.40 |
| 4      | not detected                | 0.75       | 0.654      | 85.99 ± 0.26 |
| 5      | not detected                | 0.75       | 0.659      | 87.33 ± 0.20 |
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