

Development of a method for oral administration of hydrophobic substances to *Caenorhabditis elegans*: pro-longevity effects of oral supplementation with lipid-soluble antioxidants

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Noriko Kashima, Yukiko Fujikura, Tomomi Komura, Shogo Fujiwara, Miyuki Sakamoto, Keiji Terao & Yoshikazu Nishikawa

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1 **Development of a method for oral administration of hydrophobic**
2 **substances to *Caenorhabditis elegans*: pro-longevity effects of oral**
3 **supplementation with lipid-soluble antioxidants**

4

5 **Noriko Kashima · Yukiko Fujikura · Tomomi Komura · Shogo Fujiwara · Miyuki**
6 **Sakamoto · Keiji Terao · Yoshikazu Nishikawa**

7

8 N. Kashima · Y. Fujikura · T. Komura · S. Fujiwara · M. Sakamoto · Y. Nishikawa (✉)

9 Department of Food and Human Health Sciences, Graduate School of Human Life Science,
10 Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan

11 e-mail: nisikawa@life.osaka-cu.ac.jp

12 Tel. & Fax: +81 6-6605-2910

13

14 K. Terao

15 CycloChem Co., Ltd, KIBC 654R, 5-5-2, Kobe 650-0047, Japan

16

1 **Abstract** Methods for quantitative oral administration of various substances to
2 *Caenorhabditis elegans* are needed. Previously, we succeeded in oral administration of
3 hydrophilic substances using liposomes. However, an adequate system for delivery of
4 hydrophobic chemicals was not available. In this study, we developed a method for oral
5 administration of lipid-soluble substances to *C. elegans*. γ -cyclodextrin (γ CD), which delivers
6 hydrophobic chemicals, was used to make micro-particles of inclusion compounds that can be
7 ingested by bacteriophagous nematodes, which do not distinguish these micro-particles from
8 their food bacteria. Successful oral delivery of the hydrophobic fluorescent reagent 3,3'-
9 dioctadecyloxacarboyanine perchlorate into the intestines of *C. elegans* was observed. Oral
10 administration of the hydrophobic antioxidants tocotrienol, astaxanthin, or γ -tocopherol,
11 prolonged the nematode lifespan; tocotrienol rendered them resistant to infection with the
12 opportunistic pathogen *Legionella pneumophila*. In contrast, older conventional delivery
13 methods that involve incorporation of chemicals into the nematode growth medium or
14 pouring chemicals onto the plate produced weaker fluorescence and no longevity effects. Our
15 method efficiently and quantitatively delivers hydrophobic solutes to nematodes, and a
16 minimum effective dose was estimated. In combination with our liposome method, this γ CD
17 method expands the usefulness of *C. elegans* for the discovery of functional food factors and
18 for screening drug candidates.

19

20 **Keywords** Longevity · Nematodes · Oral administration · Antioxidant · Cyclodextrin · Innate
21 immunity

1 **Introduction**

2

3 *Caenorhabditis elegans* is a free-living bacteriophagous nematode that plays an important role
4 in biological research. Despite increased use of *C. elegans* in a variety of studies, no efficient
5 method for oral administration of chemicals exists. Previously, we succeeded in oral
6 administration of hydrophilic substances using liposomes (Shibamura et al., 2009). However,
7 an adequate system for delivering hydrophobic chemicals is unavailable. Currently, these
8 chemicals are generally dissolved in the nematode growth medium (NGM) using dimethyl-
9 sulfoxide or ethanol (Ishii et al., 2004; Pun et al., 2010). Alternatively, the solution is spread
10 onto the surface of NGM with the *Escherichia coli* strain OP50 (OP50), which is the
11 international standard food of nematodes (Gruber et al., 2007; Srivastava et al., 2008).
12 However, estimating the amount of chemicals taken into nematodes with these methods is
13 difficult unless the worms are physico-chemically analyzed. Further, the amount of organic
14 solvents must be increased when higher amounts of hydrophobic chemicals need to be added
15 to the NGM.

16 We hypothesized that nematodes could efficiently ingest hydrophobic chemicals if they
17 were made into particles similar to bacteria, as we previously reported (Shibamura et al.,
18 2009). Here, we used γ -cyclodextrin (γ CD) to deliver hydrophobic substances. γ CD forms
19 inclusion compounds with hydrophobic molecules due to the unique nature of its structure.
20 γ CD is topologically represented as a toroid in which the interior is considerably less
21 hydrophilic than the aqueous environment and thus can host other hydrophobic molecules. In
22 contrast, the exterior is sufficiently hydrophilic to render the complexes water soluble. These
23 properties enable inclusion compounds containing γ CD and hydrophobic molecules to
24 penetrate tissues and release biologically active compounds (Bhagavan et al., 2007). The
25 fluorescent reagent 3,3'-dioctadecyloxycarbocyanine perchlorate (DiO) was used to test oral

1 administration of lipid-soluble substances to *C. elegans*. We compared the efficiency of γ CD-
2 mediated delivery with conventional methods.

3 Dietary supplements of antioxidants are reported to have positive effects on longevity
4 (Brown et al., 2006; Kampkötter et al., 2008; Melov et al., 2000; Wilson et al., 2006; Wu et al.,
5 2002), but other studies have reported controversial results (Bass et al., 2007; Goldstein et al.,
6 1993; Keaney et al., 2004; Larsen and Clarke, 2002). As an example of application of our
7 method, hydrophobic antioxidants were administered using both the new and conventional
8 methods to compare the effect on the nematode lifespan and on host defense against bacterial
9 infection. Using our new method, we showed clear effects of the hydrophobic antioxidants
10 tocotrienols astaxanthin and γ -tocopherol on the lifespan and host defense of *C. elegans*.

11

12

13 **Materials and methods**

14

15 **Ingestion of γ CD inclusion compounds**

16 *C. elegans* Bristol strain N2 worms were propagated on NGM with standard techniques using
17 OP50 as food bacteria (Stiernagle, 1999). Inclusion compounds of γ CD and hydrophobic
18 chemicals were prepared as shown in Fig. 1. To monitor nematode ingestion of γ CD (MW
19 1297) bound to hydrophobic chemicals, the lipid-soluble fluorescent compound DiO (MW
20 881.7) was used. A sterile γ CD solution was prepared by filtering a nearly saturated γ CD (230
21 mg/ml) solution. Sterile DiO ethanol solution (100 μ g/ml) was prepared by filtration through
22 an organic solvent-resistant filter (SLLG013SL, Millipore, Carrigtwohill, Ireland). One
23 milliliter γ CD solution was mixed with 0.1 ml DiO solution and stirred for 12-24 h with a
24 rotary converter. The solid complex (inclusion compounds) was collected by centrifugation
25 and weighed. The amount of DiO contained in the compounds was measured based on the

1 fluorescence intensity. Finally, the inclusion compound (approximately 14 mg wet weight)
2 containing 8.0 μg DiO was added to M9 buffer and vortexed so that the nematodes could
3 ingest the compounds. For comparison, 80 μl DiO ethanol solution was dissolved in a
4 peptone-free NGM (mNGM) plate (10.0 ml mNGM, 5 cm diameter) directly, or the solution
5 was poured onto the mNGM plate with OP50 (10 mg/plate). Nematodes were fed on OP50 *ad*
6 *libitum*, and then collected at various time points, counted and washed three times with M9
7 buffer. The nematodes were placed in a 0.65-ml graduated microtube (Scientific Specialties,
8 Lodi, CA, USA) containing 20 μl M9 buffer and mechanically disrupted with a microtube
9 pestle. The volume was adjusted to 100 μl with M9 buffer, and fluorescence intensity of the
10 supernatant was measured with a spectrofluorophotometer (Wallac 1420 ARVOSx,
11 PerkinElmer, Waltham, MA, USA). Standard curves were generated by plotting fluorescence
12 intensity against the concentration of DiO. The amount of DiO ingested and absorbed by a
13 worm was calculated by dividing the total DiO recovered from the worms by the number of
14 worms.

15

16 **Longevity effect**

17 After hatching, nematodes were grown on OP50 for 3 days, and then the adult worms
18 were divided into groups that were supplemented with vitamin E (Oryza Oil & Fat Chemical,
19 Ichinomiya, Aichi, Japan). The product was composed of 4.9% α -tocotrienol (T3), 0.5% β -T3,
20 61.3% γ -T3, 4.2% δ -T3, 1.2% α -tocopherol, 0.6% β -tocopherol, 4.5% γ -tocopherol, and 0.7%
21 δ -tocopherol; the total amount of T3s was more than 70% of the product, whereas tocopherols
22 comprised 7%. The inclusion compound was prepared as described above and spread onto
23 mNGM plates so that each plate contained 26, 86, or 259 μg T3s; these amounts were based
24 on the assumption that all T3s were included in the inclusion compound by the addition of a
25 sufficient amount of γ CD. Astaxanthin (Wako, Tokyo, Japan) or γ -tocopherol (Sigma, St.

1 Louis, MO, USA) was also used as another well-known antioxidant.

2 Each group of 30 worms was added to a plate and incubated at 25 °C. Live and dead
3 worms were determined every 24 h. A worm was considered dead when it failed to respond to
4 gentle touch with a worm picker. Worms that died from getting stuck to the wall of the plate
5 were not scored. Worms were transferred every other day to fresh plates to avoid
6 contamination from progeny. Each assay was carried out in duplicate and repeated two or
7 three times to confirm the reproducibility. The same amount of γ CD only was administered to
8 control worms.

9 Mean life span was estimated using the formula (Wu et al., 2006):

$$10 \quad \text{MLS} = \frac{1}{N} \sum_j \frac{x_j + x_{j+1}}{2} d_j$$

11 where d_j is the number of worms that died in the age interval (x_j, x_{j+1}), and N is the total
12 number of worms. The standard error of the mean life span estimate was calculated using the
13 equation:

$$14 \quad \text{SE} = \sqrt{\frac{1}{N(N-1)} \sum_j \left(\frac{x_j + x_{j+1}}{2} - \text{MLS} \right)^2 d_j.}$$

15 Maximum life span was calculated as the mean life span of the longest-living 15% of each
16 group.

17 Nematodes were also examined for lipofuscin, the so-called “age pigment” that
18 accumulates with aging, with fluorescence microscopy (Gerstbrein et al., 2005).

19

20 **Effect of tocotrienols on innate immunity**

21 After hatching, nematodes were grown on OP50 for 3 days. The adult worms were then
22 divided into two groups that were given γ CD only or were supplemented with a γ CD inclusion
23 compound containing T3s. Seven- or 8-day-old worms were fed *Salmonella enterica* subsp.

1 *enterica* serovar Enteritidis strain SE1 or *Legionella pneumophila* serogroup 1 strain JR32,
2 respectively, instead of OP50 as reported previously (Ikeda et al., 2007; Komura et al., 2010).
3 Their survival was measured as described above.

4

5 **Results**

6

7 Inclusion compounds (approximately 14 mg wet weight) composed of γ CD and 8.0 μ g DiO
8 were spread on the surface of a mNGM plate with OP50, and nematodes were allocated onto
9 the plate. In 20 min, the amount of fluorescent dye taken up was high compared with worms
10 administered dye by conventional methods (Fig. 2A). Nematodes showed fluorescence in the
11 lumen of the mouth to the pharynx, and beyond the pharyngeal bulb to the anus (Fig. 2B).
12 DiO fluorescence was observed not only in the intestinal lumen but in the cytoplasm of the
13 intestinal cells (Fig. 2C). Nematodes appeared to ingest the inclusion compounds in the
14 presence of OP50, showing that nematodes did not selectively avoid the compounds.

15 Using this method, tocotrienols (T3s) were administered to worms (Fig. 3). The lifespan
16 of nematodes that ingested γ CD containing T3s was longer than that of control worms
17 maintained on mNGM containing γ CD alone. Survival curves of worms maintained on
18 mNGM containing the same amount of T3s were indistinguishable from the control. γ CD-
19 mediated oral supplementation with the antioxidants did not clearly alter lipofuscin
20 accumulation (data not shown). Similarly, astaxanthin (6.0 μ g/plate) and γ -tocopherol (43
21 μ g/plate) also prolonged the lifespan of the worms (Fig. 4A and B). However, astaxanthin (6.0
22 μ g/plate) that was directly spread onto the mNGM plate failed to prolong the lifespan to the
23 same extent as the inclusion compound.

24 To study the effects of T3s on host defense, 7- or 8-day-old worms were fed *Salmonella*
25 *enterica* subsp. *enterica* serovar Enteritidis strain SE1 or *Legionella pneumophila* serogroup 1

1 strain JR32, respectively, instead of OP50. Oral supplementation with T3s failed to enhance
2 the host defense to *Salmonella* (data not shown). However, when nematodes were exposed to
3 the opportunistic pathogen *Legionella* (Komura et al., 2010), T3s protected against death from
4 the infection (Fig. 5).

5

6

7 **Discussion**

8

9 Our results show that γ CD is an excellent vehicle for quantitative oral administration of
10 hydrophobic chemicals to *C. elegans*. Because nematodes showed fluorescence in the lumen
11 of the mouth to the pharynx and beyond the pharyngeal bulb to the anus in 20 min, we
12 assessed the amount of inclusion compounds ingested by the nematodes in 20 min. When
13 inclusion compounds (14 mg wet weight) containing 8 μ g DiO (calculated based on its
14 fluorescence intensity) were spread onto mNGM with 10 mg OP50, the amount of DiO
15 ingested by a single nematode in 20 min was calculated to be 70 pg because 50 worms
16 ingested 3500 pg DiO (Fig. 2A). Thus, to intake 70 pg DiO from the inclusion compound (8
17 μ g of DiO/14 mg of compound), a single worm must ingest 120 ng of the inclusion
18 compound. The increase in DiO recovered 24 h later suggests that the fluorescent dye was
19 absorbed from the digestive tract and accumulated in the cytoplasm. The experiment with
20 DiO- γ CD inclusion compounds clearly showed that worms did not selectively ingest OP50,
21 and therefore did not distinguish the bacteria from the inclusion compounds. Assuming that
22 the worm would ingest compounds at the same rate if other chemicals were bound by γ CD,
23 this method enables us to estimate the amount of test substances ingested by a worm over a
24 particular time. It is easy to determine if worms ingested an inclusion compound as well as
25 they ingested DiO- γ CD inclusion bodies by counting the number of worms remaining in both

1 suspensions spotted separately onto mNGM plates in a preparatory experiment.

2 We showed that DiO- γ CD inclusion compounds were ingested by worms. Then, lipid-
3 soluble antioxidants were used to examine if the γ CD could carry and release chemicals that
4 retain their function. The lifespan of nematodes that ingested γ CD containing T3s was longer
5 than that of control worms. As described above, a worm ingested 120 ng of the inclusion
6 compound when 14 mg of the inclusion compound was present with OP50 on mNGM. Based
7 on the rule-of-three calculation, we estimated that a worm would ingest 57 ng out of 6.6 mg
8 of T3- γ CD inclusion compound in 20 min. Because 6.6 mg of T3- γ CD inclusion compound
9 included at most 86 μ g of T3 if we assumed that all added T3 was included, a worm would
10 intake 740 pg of T3 included in the 57 ng of the inclusion compounds, proportionally. Thus,
11 our method efficiently delivered hydrophobic antioxidants to nematodes with significant
12 effects on lifespan. The clear pro-longevity effect of T3 was consistent with a previous report
13 (Adachi and Ishii, 2000). Because our method is very efficient at delivering hydrophobic
14 antioxidants to nematodes, significant pro-longevity effects were observed at a concentration
15 of 86 μ g per mNGM plate, which is a 10-fold lower concentration than that used by Adachi
16 and Ishii (2000). Oral administration of astaxanthin (6 μ g/10 ml of mNGM plate) and γ -
17 tocopherol (43 μ g/10 ml of mNGM plate) also prolonged the lifespan at a very low dose
18 compared to previous reports in which 0.1-1 mM (59.7-597 μ g/ml) astaxanthin or 200 μ g/ml
19 γ -tocopherol were added to the medium (Yazaki et al., 2011; Zou et al., 2007). This delivery
20 mechanism should therefore facilitate clarification of the effects of hydrophobic compounds
21 such as T3 on longevity in future studies.

22 The age-dependent accumulation of lipofuscin in the intestinal cells of worms has been
23 demonstrated (Klass, 1977), but antioxidants and lifespan extension are not always associated
24 with reduction in this age pigment (Braeckman et al., 2002; Kampkötter et al., 2007). T3 may
25 play major roles in tissues other than intestinal cells, resulting in increased longevity.

1 Previously we showed that nematodes fed bifidobacteria or lactobacilli for 4 days were
2 clearly resistant to subsequent *Salmonella* infection compared with nematodes fed OP50
3 before the infection (Ikeda et al., 2007). T3s protected the worms from infection with the
4 opportunistic pathogen *Legionella*, but T3s did not prevent infection with *Salmonella* unlike
5 the lactic acid bacteria. The discrepancy is probably due to the higher virulence of *Salmonella*
6 organisms (Hoshino et al., 2008; Komura et al., 2010). Mechanisms of how T3s protected
7 worms against death from *Legionella* infection still remain to be elucidated. Protein damage
8 occurs specifically at the sites of host-pathogen interactions, and reactive oxygen species
9 produced by the host are a source of protein damage during infection (Mohri-Shiomi and
10 Garsin, 2008). T3s may reverse reactive oxygen species-mediated protein damage due to the
11 opportunistic pathogen *Legionella*, whereas the lactic acid bacteria probably enhance the
12 endogenous host defense.

13 γ CD is theoretically likely to cause cholesterol depletion by forming inclusion compounds
14 with cholesterol. Cholesterol depletion can cause brood size reduction and internal egg
15 hatching, so-called worm bagging. However, no effects on the lifespan or brood size were
16 observed when we administered saturated γ CD solution (50 μ l/plate) to nematodes. In
17 addition, our method involves administration of γ CD after it has been transformed into
18 inclusion bodies with chemicals. This is probably the reason why the γ CD did not cause
19 cholesterol depletion.

20 A few limitations of γ CD application are as follows: 1) a few organic solvents, methylene
21 chloride for example, cannot be used because they form inclusion compounds with γ CD,
22 inhibiting the reaction between γ CD and test chemicals; 2) some test chemicals would be
23 perfectly included within the cavity of γ CD and be dissolved in water without forming
24 insoluble particles; and 3) some chemicals that are larger than the γ CD cavity cannot be
25 included in γ CD inclusion bodies.

1 In conclusion, γ CD is an excellent vehicle for oral delivery of hydrophobic substances to
2 *C. elegans*. Using this method, oral supplementation with antioxidants prolonged the lifespan
3 of worms more efficiently than conventional delivery methods. In combination with our
4 liposome method (Shibamura et al., 2009), this γ CD method provides an alternative choice to
5 administer chemicals orally to *C. elegans*, particularly when test chemicals are limited or the
6 amount of organic solvents must be restricted. Our method will expand the usefulness of
7 nematodes not only in biogerontology but also for screening drugs, health-promoting
8 chemicals, and toxic substances.

9

10

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12

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18

19

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2

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8
9

1 **Figure legends**

2

3 **Fig. 1.** Flow chart of the system to prepare γ CD inclusion compounds

4

5 **Fig. 2.** Nematode fluorescence after oral administration of DiO. (A) The amount of DiO
6 recovered from worms. Bars indicate the average of five experiments. The amount of
7 fluorescent dye recovered from worms orally administered with DiO- γ CD inclusion
8 compounds was significantly higher than that from the other two groups in which DiO was
9 spread onto or dissolved into the medium. * $p < 0.05$, ** $p < 0.01$. At 40 min, only the
10 difference between DiO- γ CD and DiO in NGM was significant. (B) A nematode fed OP50
11 and γ CD bound with DiO shows clear fluorescence along the digestive tract. (C) The
12 intestinal lumen is between the arrows. Faint fluorescence is visible in the cytoplasm of
13 intestinal cells.

14

15 **Fig. 3.** Survival curves of nematodes supplemented with γ CD-tocotrienol inclusion
16 compounds. Young adult worms were divided into groups that were supplemented with T3-
17 enriched vitamin E. The inclusion compound was spread onto mNGM plates so that each
18 plate contained 26, 86, or 260 μ g T3s. The same amount of γ CD only was administered to
19 control worms and those maintained on mNGM containing 86 μ g T3s. Nematode survival
20 was calculated with the Kaplan-Meier method, and survival differences were tested for
21 significance using the log rank test. * $p < 0.05$, *** $p < 0.001$, compared to the control. The
22 mean lifespans (in days) of worms supplemented with 26, 86, or 259 μ g T3s were 16.1 ± 0.43
23 (8.6%), 18.0 ± 0.46 (21%), and 16.7 ± 0.35 (12%), respectively. The numbers in parentheses
24 are the percent differences in the mean relative to control. Mean lifespans of control worms
25 and worms supplemented with 86 μ g T3s with the conventional method were 14.8 ± 0.39 and

1 14.5 ± 0.41 (-2.6%) days, respectively. Similarly, the maximum lifespans of worms
2 supplemented with 26, 86, or 259 µg T3s were 19.6 ± 0.13 (3.5%), 22.3 ± 0.24 (18%), and
3 21.1 ± 0.29 (11%), respectively. Maximum lifespans of control worms and worms
4 supplemented with 86 µg T3s with the conventional method were 19.0 ± 0.38 and 19.6 ± 0.29
5 (3.2%) days, respectively.

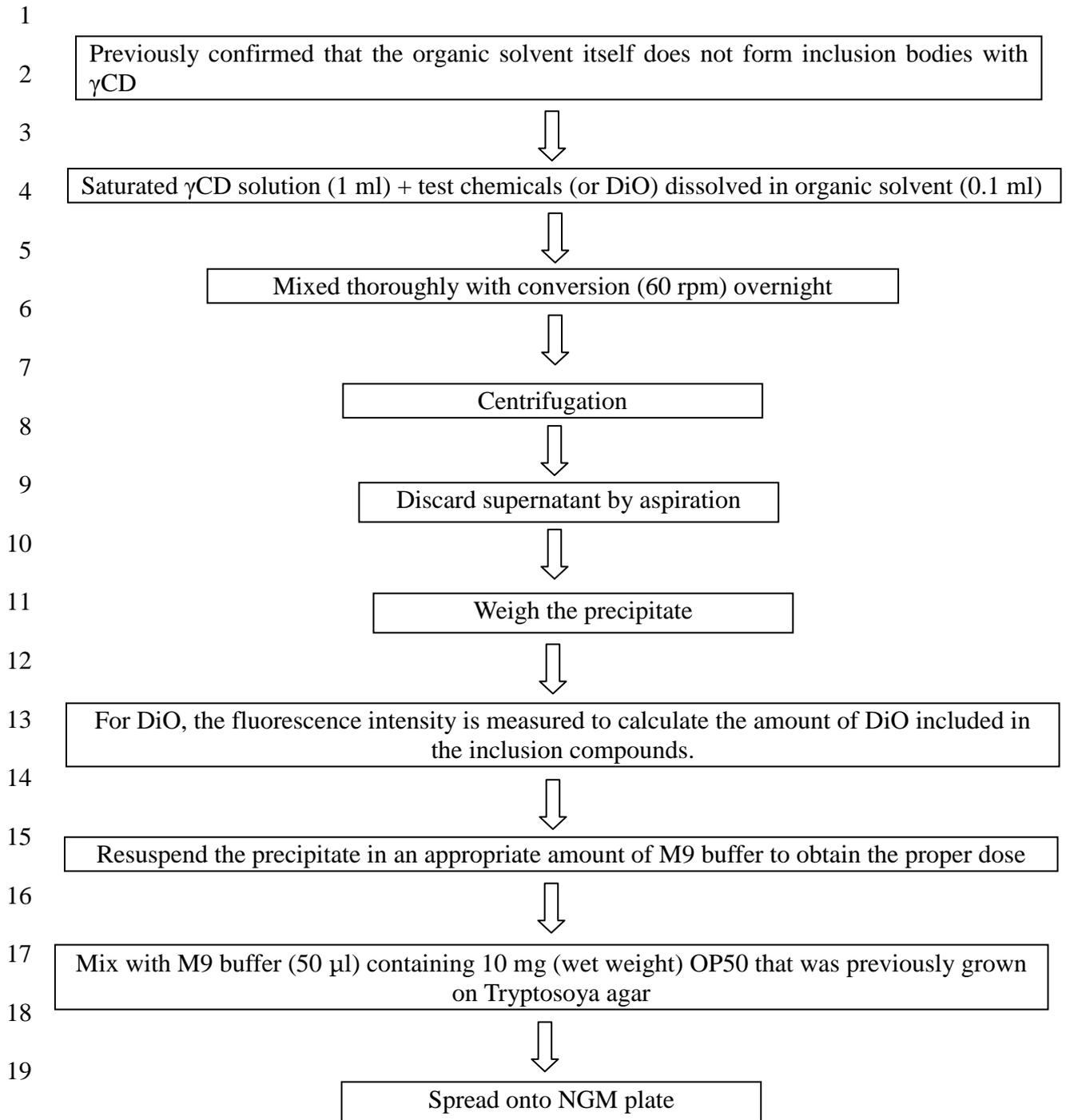
6

7 **Fig. 4.** Survival curves of nematodes supplemented with γ CD-antioxidant inclusion
8 compounds. (A) Young adult worms were divided into groups that were supplemented with
9 6.0 µg astaxanthin as the inclusion compound (AX- γ CD) or by directly spreading the DMSO
10 solution (AX-DMSO). The same amount of DMSO was administered to a group of worms to
11 compare with the control worms. Nematode survival was calculated with the Kaplan-Meier
12 method, and survival differences were tested for significance using the log rank test. * $p < 0.05$,
13 *** $p < 0.001$, compared to the control. The mean lifespans (in days) of the groups given AX-
14 γ CD, AX-DMSO, and DMSO were 20.9 ± 0.59 (20.8%), 18.6 ± 0.55 (7.5%), and 17.7 ± 0.63
15 (2.3%), respectively. The numbers in parentheses are percent differences in the mean relative
16 to control. The mean lifespan of the control was 17.3 ± 0.64 days. Similarly, the maximum
17 lifespans (in days) of each group were 25.9 ± 0.3 (14.6%), 24.4 ± 0.77 (8.0%), and 23.9 ±
18 0.53 (5.8%), respectively. The maximum lifespan of the control was 22.6 ± 0.48 days. (B)
19 Young adult worms supplemented with 43 µg γ -tocopherol as the inclusion compound (γ TP-
20 γ CD) survived longer than control worms ($p < 0.05$). The mean lifespan (in days) was 18.5 ±
21 0.49 (10.1%), whereas that of the control was 16.8 ± 0.39. The numbers in parentheses are
22 percent differences in the mean relative to control. Similarly, the maximum lifespans of
23 worms treated with γ TP- γ CD and control worms were 25.5 ± 0.44 (12.3%) and 22.7 ± 0.43,
24 respectively.

25

1 **Fig. 5.** Effect of supplementation with T3s on the survival of nematodes infected with
2 *Legionella pneumophila*. Nematodes supplemented with T3s were significantly more resistant
3 to the pathogen than controls (**p < 0.01). The mean survival times of worms after
4 *Legionella* infection were 13.5 ± 0.23 days for control worms and 15.0 ± 0.32 days for those
5 supplemented with 86 μg T3s. The percent difference in the mean relative to control was 11%.
6 Similarly, maximum survival times were 17.1 ± 0.46 days for control worms and 19.6 ± 0.72
7 (14%) days for those supplemented with 86 μg T3s.

8



23 Fig. 1

24

