OECD GUIDELINE FOR TESTING OF CHEMICALS

PROPOSAL FOR A NEW GUIDELINE 302D

Inherent Biodegradability - Concawe Test

INTRODUCTION

1. This method is based on International Organization for Standardization (ISO) 14593: "Carbon dioxide (CO_2) Headspace Biodegradation Test" (1) and provides a test for assessing the inherent aerobic biodegradability of organic substances. It is particularly useful for testing insoluble and/or volatile materials, and was successfully ring tested in 1996/97 using a formulated hydraulic fluid, hexadecane, di-isotridecyl adipate and two mineral base oils (2).

PRINCIPLE OF THE TEST

2. The test substance is incubated in a buffered, mineral salts medium which has been inoculated with a mixed population of micro-organisms. In order to enhance the biodegradative potential of the inoculum, it is pre-exposed to the test substance using a regime based on methods described in US Environmental Protection Agency Test Guideline § 796.3100 (3) (See paragraphs 20-23). The test is performed in sealed bottles with a headspace of air that provides a reservoir of oxygen (O_2) for aerobic biodegradation. CO_2 evolution from the ultimate aerobic biodegradation of the test substance is determined by measuring the inorganic carbon (IC) produced in the test bottles over that produced in blanks which contain inoculated medium only. The ultimate aerobic biodegradation is the breakdown of an organic chemical by micro-organisms in the presence of O_2, resulting in the production of CO_2, water, mineral salts (i.e. mineralisation) and microbial cellular constituents (biomass). The extent of biodegradation is then expressed as a percentage of the theoretical maximum IC production (ThIC), based on the quantity of test substance (as total organic carbon) added initially. ThIC is analogous to the term $ThCO_2$ used in OECD 301 B: CO_2 evolution (modified Sturm) test (4).

3. Dissolved organic carbon (DOC) removal (water-soluble substances only) and/or the extent of primary biodegradation of the test substance can also be measured. Primary biodegradation is the structural change (transformation) of an organic chemical by micro-organisms resulting in the loss of a specific property.

INFORMATION ON THE TEST SUBSTANCE

4. The organic carbon content (% w/w) of the test substance needs to be known either from its chemical structure or by measurement. For volatile test substances, a measured or calculated Henry's law constant is helpful for determining a suitable headspace to liquid ratio. Information on the toxicity of the test substance to bacteria is useful for selecting an appropriate test concentration and for interpreting results showing poor biodegradability. As this test is usually performed only after failure to pass a test for ready.

APPLICABILITY OF THE METHOD

5. The test is applicable to water soluble and insoluble test substances. Using the recommended headspace to liquid ratio of 1:2, volatile substances with a Henry's law constant of up to 50 Pa m^3 mol^{-1} can be tested as the proportion of test substance in the headspace will not exceed 1% (5). A smaller headspace volume may be used when testing more volatile substances. However, users must ensure that the headspace to liquid ratio and the test substance concentration are such that sufficient O_2 is available to allow complete aerobic biodegradation to occur (e.g. avoid using a high substrate concentration and a small headspace volume). Guidance on this matter can be found in (6).

REFERENCE SUBSTANCES

6. In order to check the test procedure, a reference substance of known biodegradability should be tested in parallel. For this purpose, *n*-hexadecane or a rapeseed oil with a low (≤ 2% w/w) erucic acid content ("Canola oil"), are recommended when testing insoluble substances. Sodium benzoate is recommended for water-soluble test substances. Biodegradation of these substances must reach ≥60% ThIC by the end of the test.

7. To demonstrate the increased biodegradative power of the test over a ready biodegradability test, di-isotridecyl adipate (DITA) can be used as a more difficult to biodegrade reference substance. DITA is typically biodegraded by only around 30% ThIC after 28 days with an unexposed inoculum (e.g. in OECD 301 B) but can be mineralised by 40 - 80% ThIC in this test. DITA is a reference oil (RL 130) for the CEC L-33-A-93 biodegradability test and details on how to obtain it can be found in (7).

REPRODUCIBILITY

8. Based on the CONCAWE 1996/97 ring test of the method (2), the difference between two single and independent test results obtained by different operators, working in different laboratories, on the same test substance would exceed 38% only in one case in 20.

9. In the CONCAWE 1996/97 ring test of the method (2), the following results were obtained using the recommended test conditions:

Test substance (Both insoluble)	Mean Percentage biodegradation (Day 56)	Coefficient of variation (%)	Number of laboratories
n-Hexadecane	72	21%	10
Di-isotridecyl adipate (DITA)	65	21%	10

For these two insoluble test substances, the variability (as the coefficient of variation) between replicates in the same test run (i.e. replicability) was <10% for laboratories that were experienced in using the method.

DESCRIPTION OF THE METHOD

Apparatus

10. Normal laboratory apparatus and:

- pH meter;
- analytical instruments such as GLC, HPLC, TLC equipment, including the appropriate detection systems for analysing radiolabelled and non-labelled substances or inverse isotopes dilution method;
- glass serum bottles, sealed with butyl rubber stoppers and crimp-on aluminium seals. The recommended size is '125 ml' which in fact have a total volume of around 160 ml;
- carbon analyser or other instrument(s) (e.g. gas chromatograph) for measuring inorganic carbon;
- syringes of high precision for gaseous and liquid samples;
- orbital shaker in a temperature-controlled environment;
- a supply of CO_2-free air - this can be prepared by passing air through soda lime granules or by the use of an 80% N_2 / 20% O_2 gas mixture;
- membrane filtration device of 0.20 - 0.45 µm porosity (optional);
- organic carbon analyser (optional).

Reagents

11. Use analytical grade reagents throughout.

Water

12. Distilled or de-ionised water should be used containing ≤1 mg/L as total carbon. This represents ≤5% of the initial organic carbon content introduced by the recommended dose of the test substance.

Stock solutions for the mineral salts medium

13. The stock solutions and the mineral salts medium are similar to those employed in ISO 14593 (1) and OECD 301 ready biodegradability tests (4). However, it should be noted that solution (a) in this method contains a higher concentration of ammonium chloride to prevent the possibility of biodegradation becoming nitrogen-limited.

14. Stock solutions should be stored under refrigeration and disposed of after six months, or earlier if there is evidence of microbial growth or precipitation.

15. Prepare the following stock solutions:

(a) Potassium dihydrogen phosphate (KH_2PO_4) 8.50g
Dipotassium hydrogen phosphate (K_2HPO_4) 21.75g
Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) 33.40g
Ammonium chloride (NH_4Cl) 2.00g

Dissolve in water and make up to 1 litre. The pH of this solution should be 7.4 (± 0.2). If this is not the case, then prepare a new solution.

(b) Calcium chloride dihydrate ($CaCl_2.2H_2O$) 36.40g

Dissolve in water and make up to 1 litre.

(c) Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) 22.50g

Dissolve in water and make up to 1 litre.

(d) Iron (III) chloride hexahydrate ($FeCl_3.6H_20$) 0.25g

Dissolve in water and make up to 1 litre and add one drop of concentrated hydrogen chloride.

Preparation of the mineral salts medium

16. Mix 10ml of solution (a) with approximately 800 ml water (paragraph 12), then add 1 ml each of solutions (b), (c) and (d) and make up to 1 litre with water (paragraph 12).

Inoculum

17. The test uses a composite microbial inoculum, derived from soil and a wastewater treatment plant, that has been pre-exposed to the test substance. In order to maximise the biodegradation potential of the test, samples can be taken from locations where pre-exposure to the test substance or similar materials may have already occurred (e.g. contaminated soil, industrial biotreater).

Soil

18. Clear the soil surface of leaf litter and collect a sample to a depth of up to 20 cm below the soil surface. Transport in a loosely-tied black polythene bag. Remove stones, plant remains and invertebrates from the soil and sieve through a 2 mm mesh (if the soil is too wet to sieve immediately, then partially air dry to facilitate sieving). If the sieved soil is not required immediately, then it can be stored at 2 to 4°C in a loosely-tied black polythene bag for up to one month.

Activated sludge

19. Collect activated sludge from a wastewater treatment plant and keep aerated at 20 ± 1°C until use (this should be within two days of collection).

Pre-exposure procedure

20. To 1 litre mineral salts medium, add 1 g prepared soil, 2 ml activated sludge and 50 mg/L yeast extract (e.g. Oxoid, Difco). Adjust the pH (if necessary) to 7.4 ± 0.2 and dispense 100 ml aliquots into conical flasks of 250 to 300 ml volume. Keep the suspension well mixed while dispensing. Add 4 mg/L as carbon of test substance to replicate flasks. The number required will depend on the volume of inoculum needed to perform the test. Close the flasks with stoppers that will allow gaseous exchange (e.g. foam stoppers, aluminium caps) and incubate at 20 ± 1°C, with shaking or mixing at a rate sufficient to keep the flask contents well mixed and in suspension. Losses of a volatile test substance may occur during this procedure.

21. On or around Day 7 and Day 11 of incubation, make up any evaporation losses of test medium with water, adjust the pH (if necessary) to between 7.2 and 7.6 and add 8 mg/L as carbon test substance to each flask. The final addition of test substance should be made at least three days before the inoculum is used for the test.

22. On the day of the test (normally Day 14 of pre-exposure) coarse-filter the inoculum through a number 4 grade filter paper or glass wool and keep shaken until use. If two or three substances are being tested, the pre-exposed cultures for each test substance can be pooled to give a composite inoculum. This reduces the number of blanks needed and provides a microbial population that has been exposed to more than one substance.

23. The inoculum is normally used within one day. If problems with invalid (i.e. too high) blank inorganic carbon production (see paragraph 50) have been encountered, or are thought likely to occur, then one or both of the following treatments are recommended:

- aerate the inoculum overnight before use (this reduces its organic carbon content);
- before use, sparge the inoculum with CO_2-free air for about one hour, while maintaining the pH at 6.5 using orthophosphoric acid. Finally restore the pH to its original value (this reduces its inorganic carbon content).

TEST PROCEDURE

Number of bottles

24. The number of bottles needed for a test will depend on the frequency of analysis and the test duration. It should be noted that the test may have to be continued beyond the 56 days suggested in paragraph 35.

25. It is recommended that at least five bottles from sets Bottle-1, 2 and 3 (see paragraph 28) are analysed at the end of the test, to enable 95% confidence intervals to be calculated for the mean percentage biodegradation value.

Inoculated medium

26. The inoculum is used at a concentration of 10% v/v (e.g. add 100 ml inoculum to 900 ml mineral salts medium).

Preparation of bottles

27. Aliquots of inoculated medium are dispensed into replicate bottles to give a headspace to liquid ratio of 1: 2 (e.g. add 107 ml to 160 ml-capacity bottles). Other ratios may be used but see the warning given in paragraph 5.

28. Sets of bottles should be prepared containing the following :

Bottle-1: The test substance (held on a glass fibre filter, if necessary – see paragraph 30) at a recommended concentration of 20 mg/L as organic carbon;

Bottle-2: Blanks containing inoculated medium (and a glass fibre filter, if necessary) only;

Bottle-3: The reference substance at a recommended concentration of 20 mg/L as organic carbon (and a glass fibre filter, if necessary);
If there is a possibility that the test substance may undergo abiotic degradation, then also prepare sets of sterile controls.

Bottle-4: Bottle-1 plus 50 mg/L $HgCl_2$ or sterilised by some other means (e.g. by autoclaving);

If there is a possibility that the test substance may be inhibitory at the test concentration, then also prepare sets of bottles.

Bottle-5: Containing the test substance plus the reference substance at the same concentrations as used for Bottle-1 and 3.

29. Water-soluble test and reference substances are added from aqueous stock solutions.

30. Insoluble test and reference substances are added to bottles as a measured weight absorbed on a GF/A glass fibre filter (21 mm diameter). This enables an accurate weight to be dosed into each bottle and increases bioavailability. Weighing can be facilitated by preparing a stock solution of the substance in a suitable volatile solvent (e.g. 1,1,2-trichlorotrifluoroethane, dichloromethane) and adding the required volume drop-wise to a tarred filter on an analytical balance. An attempt should be made to spread the substance over the filter. The solvent is then allowed to evaporate until a stable weight (or nominal test weight) is obtained. For example, 100 µl of a 21.4 mg/mL as carbon stock solution added to a filter gives 2.14 mg carbon/filter (i.e. 20 mg/L carbon when added to 107 ml inoculated medium). The measured weight of test substance on the filter should be used to calculate the test dose (TOC in paragraph 45).

31. If the procedure given in paragraph 30 is used, then blank bottles (Bottle-2) should contain either a filter or a filter from which an equivalent volume of solvent has been evaporated (removal of the solvent should be checked by weighing).

32. Volatile test substances should be injected into sealed bottles using a microsyringe. The syringe should be weighed before and after dosing to enable an accurate test dose to be calculated.

33. All the bottles are then sealed and, if necessary, time zero analyses performed for initial IC concentration (sterile controls, Bottle-4), DOC concentration, or other determinants.

Incubation

34. The bottles are incubated in the dark or diffuse light at 20 ± 1°C, with shaking at a rate sufficient to keep the bottle contents well mixed and in suspension (e.g. 150 to 200 rpm).

Sampling

35. Replicate test (Bottle-1), blank (Bottle-2), reference (Bottle-3), and if used, inhibition control (Bottle-5) bottles should be taken for IC analysis periodically to obtain the biodegradation curves. Normally this is done at least once a week. At the end of the test, the sterile controls (Bottle-4), if used, should also be analysed. The test normally runs until biodegradation reaches a plateau - as a guide, users should plan for an eight-week test. However, the test should be continued if biodegradation is still occurring on Day 56, or if the maximum level of biodegradation is required. In these circumstances, it is not advisable to continue the test beyond three months.

Inorganic carbon (IC) analysis

36. CO_2 production in the bottles is determined by measuring the increase in the concentration of inorganic carbon (IC). There are two methods available for measuring the amount of IC produced in the test, and these are described below. As the methods can give slightly different results, only one should be used in a test run.

37. Method (a) (paragraphs 38-41) is recommended if the liquid medium is likely to contain the remnants of a GF/A filter and/or insoluble test substance. This analysis can be performed using a gas

chromatograph if a carbon analyser is unavailable. It is important that the bottles should be at or close to the test temperature of 20 ± 1°C when the headspace gas is analysed. Method (b) (paragraphs 42-44) can be easier for laboratories using carbon analysers to measure IC. It is important that the sodium hydroxide solution used to convert CO_2 to carbonate is either freshly prepared or its IC content is known so that this can taken into account when calculating the test result (see paragraph 46).

Method (a): acidification to pH <3

38. Calibrate the IC analyser using an appropriate IC standard (e.g. 1% w/w CO_2 in N_2). Inject concentrated (≥85% w/v) orthophosphoric acid through the septum of each test bottle sampled to lower the pH of the medium to <3 (e.g. add 1 ml to 107 ml test medium). Replace the bottles on the shaker. After shaking for one hour at the test temperature (20 ± 1°C), remove the bottles from the shaker, withdraw aliquots (e.g. 1 ml) of gas from the headspace of each bottle and inject into the IC analyser. Record the measured IC concentration (mg/L carbon).

39. The principle of this method is that after acidification to pH <3 and equilibration at 20°C, the equilibrium constant for the distribution of CO_2 between the liquid and gaseous phases in the test bottles is 1.0 (2)(5)(8). This should be demonstrated for the test system at least once (e.g. at the end of the test) as follows: set up bottles containing 5 and 10 mg/L as IC using a solution of anhydrous sodium carbonate (Na_2CO_3) in CO_2-free water (prepared by acidifying water to pH 6.5 with concentrated orthophosphoric acid, sparging overnight with CO_2-free air and raising the pH to neutrality with alkali).

40. Ensure that the ratio of the headspace volume to the liquid volume is the same as in the tests (e.g. 1 : 2). Acidify and equilibrate as described in paragraph 38, and measure the IC concentrations of both the headspace and liquid phases. Check that the two concentrations are the same within experimental error.

41. If DOC removal is to be measured (water-soluble test substances only), take samples of the liquid phase from separate (unacidified) bottles, membrane filter and inject into the DOC analyser. These bottles can be used for other analyses as necessary.

Method (b): conversion of CO_2 to carbonate

42. Calibrate the IC analyser using an appropriate standard - for example, a solution of sodium bicarbonate ($NaHCO_3$) in CO_2-free water (see paragraph 39) in the range 0 to 20 mg/L as IC. Inject 7 M sodium hydroxide (e.g. 1 ml to 107 ml medium) through the septum of each test bottle sampled and shake for one hour at the test temperature (20 ± 1°C). Remove the bottles from the shaker, allow to settle and withdraw, by syringe, suitable volumes (e.g. 50 to 200 µl) from the liquid phase in each vessel. Inject the samples into the IC analyser and record the concentration of IC.

43. The principle of this method is that after the addition of alkali and shaking, the concentration of IC in the headspace is negligible. This should be checked for the test system at least once (e.g. at the end of the test) by using IC standards, adding alkali and equilibrating, and measuring the concentration of IC in both the headspace and liquid phases (see paragraph 39).

44. If DOC removal is to be measured (water-soluble test substances only), take samples of the liquid phase from separate bottles (no alkali) and inject into the DOC analyser. These bottles can be used for other analyses as necessary.

DATA AND REPORTING

Calculation of results

45. Assuming 100% mineralisation of the test substance to CO_2, the theoretical maximum IC production (ThIC) in excess of that produced in the blanks equals the amount of total organic carbon (TOC) from the test substance added to each bottle at the start of the test. For insoluble substances, TOC should be calculated from the measured weight of test substance absorbed on the filter and its organic carbon content. For volatile substances, TOC should be calculated from the measured weight of test substance dispensed by microsyringe and its organic carbon content.
The total mass (mg) of inorganic carbon (TIC) in each bottle is:

$$TIC = (\text{mg C in the liquid} + \text{mg C in the headspace}) = (V_L \times C_L) + (V_H \times C_H) \quad \text{Equation [1]}$$

where :

V_L = volume of liquid in the bottle (litre);
C_L = concentration of IC in the liquid (mg/L as carbon);
V_H = volume of the headspace (litre);
C_H = concentration of IC in the headspace (mg/L as carbon).

46. The calculation of TIC for the two analytical methods used for measuring IC in this test are described below in paragraphs 48 and 49. Percentage biodegradation (*% D*) in each case is given by:

$$\%D = \frac{(TIC_t - TIC_b)}{TOC} \times 100$$

where :

TIC_t = mg TIC in test bottle at time t;
TIC_b = mean mg TIC in blank bottles at time t;
TOC = mg TOC added initially to the test vessel.

Determine *% D* for the test (Bottle-1), reference (Bottle-3) and inhibition control bottles (Bottle-5), if included, from the amounts of TIC produced up to each sampling time.

47. If there has been a significant increase in the TIC content of the sterile controls over the test period, then abiotic degradation of the test substance has occurred.

Acidification to pH <3

48. Acidification to pH <3 and equilibration results in the equalisation of the concentration of IC in the liquid and gaseous phases. Hence only the concentration of IC in the gas phase needs to be measured as $C_L = C_H$. Therefore $TIC = (V_L + V_H) \times C_H$ in Equation [1].

Conversion of CO_2 to carbonate

49. In this method calculations are performed as described in Equation 1 but the negligible amount of IC in the gaseous phase is ignored (i.e. $(V_H \times C_H)$ approximately 0 in Equation [1]).

Validity of results

50. The test is considered as valid if:

- the mean percentage biodegradation of *n*-hexadecane, the low erucic acid-containing rapeseed oil ("Canola oil") or sodium benzoate reaches at least 60% ThIC by the end of the test;
- the mean amount of IC produced from the blanks at the end of the test is ≤15% of the organic carbon added initially as the test substance to the test bottles.

If these criteria are not met, repeat the test with another inoculum and/or review procedures. For example, if high blank IC production is a problem then follow the procedures given in paragraph 23.

51. If inhibition controls were included in the test, it can be assumed that the test substance is inhibitory if the mean percentage degradation in these bottles is <25% by the end of the test. In this case, the test can be repeated with a lower concentration of test substance (e.g. 10 mg/L as carbon). However, it should note that this will reduce the precision of the method. If lower test substance concentrations are used, it is important that blank IC production is as low as possible and that the smaller amount of test substance can be dosed accurately.

Interpretation of results

52. Biodegradation ≥60% ThIC in this test demonstrates that the test substance is inherently and ultimately biodegradable under aerobic conditions.

53. Biodegradation ≤20% ThIC is an indication that the test substance is not inherently biodegradable under the conditions of this test. However, this does not rule out metabolism under different circumstances (e.g. longer pre-exposure period, higher biomass to test substance ratio).

54. Biodegradation is >20% ThIC is an indication that the test substance has inherent, primary biodegradability.

55. If more information is needed on the potential fate of the test substance, then further investigations such as DOC analysis to detect water-soluble metabolites may be useful.

Test report

56. Compile a table of *% D* for each test (Bottle-1), reference (Bottle-3) and, if included, inhibition control bottle (Bottle-5) for each day sampled. If comparable results are obtained for replicate bottles, plot a curve of mean *% D* against time. Record the amount of TIC in the blanks (Bottle-2), and in the sterile controls (Bottle-4), if included in the test. If determined, also record the levels of DOC and/or other determinants and their percentage removal.

57. Determine the mean value of *% D* in the plateau phase, or use the highest value if the biodegradation curve decreases after peaking, and report this as the "extent of biodegradation of the test substance". It is important to ensure that in the latter case the highest value is not an outlier.

58. The test report must include the following information:

Test substance:

- common name, chemical name, CAS number, structural formula and relevant physical-chemical properties;
- purity (impurities) of test substance.

Test conditions:

- reference to this Test Guideline;
- description of the test system used (e.g. volume of the vessel, head space to liquid ratio, method of stirring, etc);
- application of test substance and reference substance to test system: test concentration used and amount of carbon dosed into each test bottle, any use of solvents;
- details of the inoculum used, its pre-exposure to the test substance and any pre-conditioning (e.g. overnight aeration)
- incubation temperature;
- validation of the principle of IC analysis;
- main characteristics of the IC analyser employed (and any other analytical methods used);
- number of replicates.

Results:

- raw data and calculated values of biodegradability in tabular form and degradation curves;
- reasons for any rejection of the test results;
- any other facts that are relevant to the procedure followed;
- discussion of results.

LITERATURE

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