OECD GUIDELINES FOR THE TESTING OF CHEMICALS

PROPOSAL FOR A NEW GUIDELINE

Partition Co-efficient (n-Octanol/Water): Slow-Stirring Method

INTRODUCTION

1. $P_{OW}$ values up to $\log P_{OW}$ of 8.2 have been accurately determined by the slow-stirring method (1). Therefore it is a suitable experimental approach for the direct determination of $P_{OW}$ of highly hydrophobic compounds.

2. Other guidelines describing methods for the determination of the $n$-octanol/water partition coefficient ($P_{OW}$) are OECD guideline 107 and 117 (2)(3). The former refers to the ‘shake-flask’ method, the latter describes the determination of the $P_{OW}$ from reversed phase HPLC-retention behavior. The ‘shake-flask’ method is prone to artifacts due to transfer of octanol microdroplets into the aqueous phase. With increasing values of $P_{OW}$ the presence of these droplets in the aqueous phase leads to an increasing overestimation of the concentration of the test compound in the water. Therefore, its use is limited to compounds with $\log P_{OW} < 4$. The second method relies on solid data of directly determined $P_{OW}$ values to calibrate the relationship between HPLC-retention behavior and measured values of $P_{OW}$. In addition, a draft OECD guideline is available for determining 1-octanol/water partition coefficients of ionisable substances (4).

3. This guideline has been developed in the Netherlands. The precision of the methods in this guideline has been validated and optimized in a ring-test validation study in which 15 laboratories participated (5-4).

INITIAL CONSIDERATIONS

Significance and use

4. For inert organic substances highly significant relationships have been found between n-octanol/water partition coefficients $P_{OW}$ and their bioaccumulation in fish (5)(6)(7)(8). Moreover, $P_{OW}$ has been demonstrated to be correlated to fish toxicity as well as to sorption of chemicals to solids such as soils and sediments (10)(11). An extensive overview of the relationships has been given in reference (6).

5. A wide variety of relationships between the n-octanol/water partition coefficient and other properties of relevance to environmental toxicology and chemistry have been established. As a consequence, the n-octanol/water partition coefficient has evolved as a key parameter in the assessment of the environmental risk of chemicals as well as in the prediction of fate of chemicals in the environment.

Scope
6. The slow-stirring experiment is thought to reduce the formation of microdroplets, specifically of 1-octanol droplets in the water phase. As a consequence, overestimation of the aqueous concentration due to test substance molecules associated to such droplets does not occur. Therefore, the slow-stirring method is particularly suitable for the determination of $P_{OW}$ for substances with expected log $P_{OW}$ values of 5 and higher, for which the shake-flask method (2) is prone to yield erroneous results.

DEFINITION AND UNITS

7. The partition coefficient of a substance between water and a lipophilic solvent (n-octanol) characterizes the equilibrium distribution of the chemical between the two phases. The partition coefficient between water and n-octanol ($P_{OW}$) is defined as the ratio of the equilibrium concentrations of the test substance in n-octanol saturated with water ($C_{Ow,i}$) and water saturated with n-octanol ($C_{W,i}$).

$$K_{P_{OW,i}} = \frac{C_{Ow,i}}{C_{W,i}}$$

As a ratio of concentrations it is dimensionless. Most frequently it is given as the logarithm to the base 10 (log $P_{OW}$). $P_{OW}$ is temperature dependent and reported data should include the temperature of the measurement.

PRINCIPLE OF THE METHOD

8. In order to determine the partitioning coefficient, water, n-octanol, and the test substance are equilibrated with each other at constant temperature. Then the concentrations of the test substance in the two phases are determined.

9. The experimental difficulties associated with the formation of microdroplets during the shake-flask experiment can be reduced in the slow-stirring experiment proposed here. In the slow-stirring experiment, water, n-octanol and the test substance are equilibrated in a thermostated stirred reactor. Exchange between the phases is accelerated by stirring. The stirring introduces limited turbulence which enhances the exchange between n-octanol and water without microdroplets being formed (1).

APPLICABILITY OF THE TEST

10. The octanol/water partition coefficient is a measure of the test chemical’s activity coefficient in water relative to n-octanol (12)(13). Since the presence of substances other than the test substance might influence the activity coefficient of the test substance, the test substance should be tested as a pure substance. The highest purity commercially available should be employed for the n-octanol/water partition experiment.

11. The present method applies to pure substances that do not dissociate or associate and that do not display significant interfacial activity. It can be applied to determine the n-octanol/water partition ratio of such substances and of mixtures. When the method is used for mixtures, the n-octanol/water partition ratios determined are conditional. These octanol/water partition ratios are conditional parameters that depend on the chemical composition of the mixture tested and on the electrolyte composition employed as aqueous phase. Provided additional steps are taken, the method is also applicable to dissociating or associating compounds (paragraph 12).
12. Expert judgement is required when n-octanol-water partition ratios are determined for dissociating or associating compounds and mixtures. Due to the multiple equilibria in water and 1-octanol involved in the n1-octanol/water partitioning of dissociating substances such as organic acids and phenols, organic bases, and organometallic compounds, the n1-octanol/water partition ratio is a conditional constant strongly dependent on electrolyte composition (242)(844). Determination of the n1-octanol/water partition ratio therefore requires that pH and electrolyte composition be controlled in the experiment and reported. Expert judgement has to be employed in the evaluation of these partition ratios. Using the value of dissociation/protonation or deprotonation constant(s), suitable pH-values need to be selected, such that a partitioning ratio is determined for each ionization state. Non-complexing buffers must be used when testing organometallic compounds (844). Taking the existing knowledge on the aqueous chemistry (complexation constants, dissociation constants) into account, the experimental conditions should be chosen in such a manner that the speciation of the test substance in the aqueous phase can be estimated. The ionic strength should be identical in all experiments by employing a background electrolyte.

13. Difficulties in the test may arise in conducting the test for substances with low water solubility or high P_{OW}, due to the fact that the concentrations in the water become very low such that their accurate determination is difficult. This Guideline proposal provides guidance how to deal with this problem (14). Taking the existing knowledge on the aqueous chemistry (complexation constants, dissociation constants) into account, the experimental conditions should be chosen in such a manner that the speciation of the test compound in the aqueous phase can be estimated. The ionic strength should be identical in all experiments by employing a background electrolyte.

14. Chemical reagents should be of analytical grade or of higher purity. The use of non-labelled test substances with known chemical composition and preferably at least 99% purity or of radiolabelled test substances with known chemical composition and radiochemical purity, is recommended. In the case of short half-life tracers, decay corrections should be applied. In case of radiolabelled test substances it needs to be made sure by means of a chemical specific analytical method that the measured radioactivity is directly related to the test substance.

15. An estimate of log P_{OW} may be obtained by using commercially available software for estimation of log P_{OW}, or by using the ratio of the solubilities in both solvents.

16. Before carrying out a slow-stirring experiment for determination of P_{OW}, the following information on the test substance should be available:

(a) structural formula;
(b) suitable analytical methods for determination of the concentration of the substance in water and 1-octanol;
(c) dissociation constant(s) of ionisable substances [OECD Guideline 112] (945);
(d) aqueous solubility [OECD Guideline 105] (1046);
(e) abiotic hydrolysis [OECD Guideline 111] (1147);
(f) ready biodegradability [OECD Guideline 301] (1248);
(g) vapor pressure [OECD Guideline 104] (1349).
DESCRIPTION OF THE METHOD

Equipment and apparatus

17. Standard laboratory equipment is required, in particular, the following: including vessels to contain solutions. They have to be made from inert material such that adsorption to vessel surfaces is negligible.

- magnetic stirrers and Teflon coated magnetic stir bars are employed to stir the water phase;
- analytical instrumentation, suitable for determination of the concentration of the test substance at the expected concentrations;
- stirring-vessel with a tap at the bottom. If the estimate of log P<sub>OW</sub> exceeds six, the use of a reaction vessel of the same geometry larger than one litre has to be considered, so that a sufficient volume of water can be obtained for chemical extraction and analysis. Larger water samples can be obtained. This will result in higher concentrations in the water extract and thus a more reliable analytical determination. A schematic drawing of a glass-jacketed stirring-vessel with a volume of ca. one litre is given in the Annex. For highly hydrophobic chemicals a larger reaction vessel must be chosen such that a sufficient volume of water can be obtained for chemical extraction and analysis. The proportions of the vessel shown in the Annex have proven favorable and should be maintained when apparatus of a different size is used;
- a means for keeping the temperature constant during the slow-stirring experiment is essential for keeping the temperature constant during the slow-stirring experiment.

18. Vessels should be made from inert material such that adsorption to vessel surfaces is negligible.

Preparation of the test solutions

19. The P<sub>OW</sub> determination should be carried out with the highest purity 1-octanol that is commercially available (at least +99 %). Use analytical grade octanol at least. Purification of n<sub>1</sub>-octanol by extraction with acid, base and water and subsequent drying is recommended. In addition, distillation can be used to purify n<sub>1</sub>-octanol. Purified n<sub>1</sub>-octanol is to be used to prepare standard solutions of the test substances compounds. Water to be used in the P<sub>OW</sub> determination should be glass or quartz distilled, or obtained from a purification system (Millipore or equivalent), or HPLC-grade water may be used. Filtration through or over a 0.22 µm filter is required for distilled water, and blanks should be included to check that no impurities are in the concentrated extracts that may interfere with the test substance. If a glass fiber filter is used, it should be cleaned by baking for at least three hours at 400 °C.

20. Both solvents are mutually saturated prior to the experiment by equilibrating them in a sufficiently large vessel. This is accomplished by slow-stirring the two-phase system for two days.

21. An appropriate concentration of test substance is selected and dissolved in n<sub>1</sub>-octanol (saturated with water). The 1-octanol/water partition coefficient needs to be determined in dilute solutions in 1-octanol and water. Therefore the concentration of the test substance should not exceed 0.1M in either phase (1). The 1-octanol solutions used for the experiment must be devoid of suspended solid test substance (in the form of microcrystals).

22. The appropriate amount of test substance is dissolved in n<sub>1</sub>-octanol (saturated with water). If the estimate of log P<sub>OW</sub> exceeds five, care has to be taken that the 1-octanol solutions used for
the experiment are devoid of suspended solid test substance chemical (in the form of microcrystals). To that end, the following procedure for chemicals with an estimated value of \( \log P_{\text{OW}} > 5 \) is followed:

- the test substance compound is dissolved in 1-octanol (saturated with water);
- the solution is given time such that suspended solid substance chemical has time to settle out precipitate. During the settling period precipitation process, the concentration of the test substance compound is monitored;
- after the measured concentrations in the 1-octanol-solution have attained stable values, the stock solution is diluted with an appropriate volume of n1-octanol;
- the concentration of the diluted stock solution is measured. If the measured concentration is consistent with the dilution, the diluted stock solution can be employed in the slow-stirring experiment.

**Extraction and analysis of samples**

**Preliminary estimate of the partition co-efficient**

23. A validated analytical method should be used for the assay of test substance. The investigators have to provide evidence that the concentrations in the water saturated 1-octanol as well as in the 1-octanol saturated water phase during the experiment are above the method limit of quantification of the analytical procedures employed. Analytical recoveries of the test substance chemical from the water phase and from the 1-octanol phase need to be established prior to the experiment in those cases for which extraction methods are necessary. The analytical signal needs to be corrected for blanks and care should be taken that no carry-over of analyte from one sample to another can occur.

24. Extraction of the water phase with an organic solvent and preconcentration of extract are likely to be required prior to analysis, due to rather low concentrations of hydrophobic test substances compounds in the water phase. For the same reason it is necessary to reduce eventual blank concentrations. To that end, it is necessary to employ high purity solvents, preferably solvents for residue analysis. Moreover, working with carefully pre-cleaned (e.g. solvent washing or baking at elevated temperature) glassware can help to avoid cross-contamination.

25. An estimate of \( \log P_{\text{OW}} \) may have to be obtained from an estimation program or by expert judgment. If the value is higher than six then blank corrections and analyte carry-over need to be monitored closely. Similarly, if the estimate of \( \log P_{\text{OW}} \) exceeds six, the use of a surrogate standard for recovery correction is mandatory, so that high preconcentration factors can be reached. A number of software programs for the estimation of \( \log P_{\text{OW}} \) is commercially available\(^1\), e.g., Clog P\(^{14,20}\), KOWWIN\(^{15,21}\), ProLogP\(^{16,22}\) and ACDC log P\(^{17,23}\). Descriptions of the estimation approaches for calculation can be found in the references\(^{18-20,24,25,26}\).

26. The limit of quantification (LOQ) for determination of the test substance in 1-octanol and water are established using accepted methods. As a rule of thumb, the method limit of quantification can be determined as the concentration in water or 1-octanol that produces a signal to noise ratio of ten. A suitable extraction and pre-concentration method should be selected and analytical recoveries should also be specified. A suitable pre-concentration factor is selected in order to obtain a signal of the required size upon analytical determination.

27. On the basis of the parameters of the analytical method and the expected concentrations, an approximate sample size required for an accurate determination of the compound concentration is determined. It should be avoided that too small water samples are taken in order to obtain a sufficient

\(^1\) For example, This information is only given for the convenience of users. Other equivalent computer programmes may be used if they can be shown to produce the same results.
analytical signal. It should also be avoided taking too large water samples since otherwise there might be too little water left for the minimum number of analyses required (n = 5).

28. Quantification of the test substances occurs by comparison with calibration curves of the respective compound. The concentrations in the samples analyzed must be bracketed by concentrations of standards.

29. The test compound may need to be extracted from the aqueous phase using a suitable solvent. The analytical recovery of the test compound for octanol saturated water has to be established. For test compounds with an log $P_{OW}$ estimate higher than six a surrogate standard has to be spiked to the water sample prior to extraction in order to register losses occurring during extraction and pre-concentration of the water samples. For accurate recovery correction, the surrogates must have properties that are very close to, or identical with those of the test substance. Preferably, (stable) isotopically-labelled analogs of the substances of interest (for example, perdeuterated or $^{13}$C-labelled) are used for this purpose. If the use of radiolabelled substances is not possible, it should be demonstrated from reliable data in the literature that the physical-chemical properties of the surrogate are very close to that of the test substance. During shake-flask liquid-liquid extraction of the water phase emulsions can form. They can be reduced by addition of salt and allowing the emulsion to settle overnight. Methods used for extracting and pre-concentrating the samples need to be reported.

30. Samples withdrawn from the n-octanol phase may, if necessary, be diluted with a suitable solvent prior to analysis. Moreover, the use of surrogate standards for recovery correction is recommended for substances for which the recovery experiments demonstrated a high degree of variation in the recovery experiments (relative standard deviation > 10%).

31. The details of the analytical method need to be reported. This includes the method of extraction, pre-concentration and dilution factors, instrument parameters, calibration routine, calibration range, analytical recovery of the test compound from water, addition of surrogate standards for recovery correction, blank values, and detection limits and limits of quantification.

Performance of the Test

Optimal 1-octanol/water volume ratios

32. When choosing the water and 1-octanol volumes, the limits of quantification in 1-octanol and water, the pre-concentration factors applied to the water samples, the volumes sampled in 1-octanol and water, and the expected concentrations should be considered. For experimental reasons, the volume of 1-octanol in the slow-stirring system should be chosen such that the 1-octanol layer is sufficiently thick (>0.5cm) in order to allow for sampling of the 1-octanol phase without disturbing it.

33. Typical phase ratios used for the determinations of compounds with log $P_{OW}$ of 4.5 and higher are 20 to 50 ml of n-octanol and 950 to 980 ml of water in a one litre vessel.

Test conditions

34. During the test the reaction vessel is thermostated to reduce temperature variation to below 1 °C. The assay should be performed between 20 and 25 °C.

35. The experimental system should be protected from daylight by either performing the experiment in a dark room or by covering the reaction vessel with aluminum foil.
36. The experiment should be performed in a dust-free (as far as possible) environment.

37. The 1-octanol-water system is stirred until equilibrium is attained. In a pilot experiment the length of the equilibration period is assessed by performing a slow-stirring experiment and sampling water and 1-octanol periodically. The sampling time points should be interspersed by a minimum period of five hours.

38. Each $P_{OW}$ determination has to be performed employing at least three independent slow-stirring experiments.

**Determination of the equilibration time**

39. It is assumed that the equilibrium is achieved when a regression of the 1-octanol/water concentration ratio against time over a time span of four time points yields a slope that is not significantly different from zero at a p-level of 0.05. The minimum equilibration time is one day before sampling can be started. As a rule of thumb, sampling of substances with a log $P_{OW}$ estimate of less than five can take place during days two and three. The equilibration might have to be extended for more hydrophobic compounds. For a compound with log $P_{OW}$ of 8.23 (decachlorobiphenyl) 144 hours were sufficient for equilibration. Equilibrium is assessed by means of repeated sampling of a single vessel.

**Starting the experiment**

40. At the start of the experiment the reaction vessel is filled with 1-octanol-saturated water. Sufficient time should be allowed to reach the thermostated temperature.

41. The desired amount of test substance (dissolved in the required volume of 1-octanol saturated with water) is carefully added to the reaction vessel. This is a crucial step in the experiment, since turbulent mixing of the two phases has to be avoided. To that end, the 1-octanol phase can be pipetted slowly against the wall of the experimental vessel, close to the water surface. It will subsequently flow along the glass wall and form a film above the water phase. Avoid at all times to pour 1-octanol directly into the flask should always be avoided; or to allow drops of 1-octanol should not be allowed to fall directly into the water.

42. After starting the stirring, the stirring rate should be increased slowly. If the stirring motors cannot be appropriately adjusted the use of a transformer should be considered. The stirring rate should be adjusted so that a vortex at the interface between water and 1-octanol of 0.5 to maximally 2.5cm depth is created. The stirring rate should be reduced if the vortex depth of 2.5cm is exceeded, otherwise microdroplets of 1-octanol droplets in the water phase may be formed, leading to an overestimation of the concentration of the test substance in the water. The maximum stirring rate of 2.5cm is recommended on the basis of the findings in the ring-test validation study (5). It is a compromise between achieving a rapid rate of equilibration, while limiting the formation of 1-octanol microdroplets.

**Sampling and Sample Treatment**

43. The stirrer should be turned off prior to sampling and the liquids should be allowed to stop moving. After sampling is completed, the stirrer is started again slowly, as described above, and then the stirring rate is increased gradually.

44. The water phase is sampled from a stop-cock at the bottom of the reaction vessel. Always discard the dead volume of water contained in the taps (approximately 5 ml in the vessel shown in the Annex). The water in the taps is not stirred and therefore not in equilibrium with the bulk. Note
the volume of the water samples, and make sure that the amount of test substance present in the discarded water is taken into account when setting up a mass balance. Evaporative losses should be minimized by allowing the water to flow quiescently into the separatory funnel, such that there is no disturbance of the water/1-octanol layer. Avoid evaporative losses due to turbulent water flow by allowing the water to flow in a laminar flow into the separation funnel.

45. 1-Octanol samples are obtained by withdrawing a small aliquot (ca 100 µL) from the 1-octanol layer with a (disposable) glass pipet or 100 microliter all glass-metal syringe. Care should be taken not to disturb the boundary. The volume of the sampled liquid is recorded. A small aliquot is sufficient, since the 1-octanol sample will be diluted.

46. Unnecessary sample transfer steps should be avoided. To that end the sample volume should be determined gravimetrically. In case of water samples this can be achieved by collecting the water sample in a separation funnel that contains already the required volume of solvent.

47. The regression used to demonstrate attainment of equilibrium should be based on the results of at least four determinations of $C_W / C_O$.

DATA AND REPORTING

4748. According to the present guideline, $P_{OW}$ is determined by performing three slow-stirring experiments (three experimental units) with the compound under investigation employing identical conditions. In each slow stirring experiment five observations of the ratio $C_O / C_W$ are obtained. The regression used to demonstrate attainment of equilibrium should be based on the results of at least four determinations of $C_O / C_W$ at consecutive time points. This allows for calculating variance as measure of the uncertainty of the average value obtained by each experimental unit.

48. The $P_{OW}$ can be characterized by the variance in the data obtained for each experimental unit. This information is employed to calculate the $P_{OW}$ as the weighted average of the results of the individual experimental units. To do so, the inverse of the variance of the results of the experimental units is employed as weight. As a result, data with a large variation (expressed as the variance) and thus with lower reliability have less influence on the result than data with a low variance.

49. Analogously, the weighted standard deviation is calculated. It characterizes the repeatability of the $P_{OW}$ measurement. A low value of the weighted standard deviation indicates that the $P_{OW}$ determination was very repeatable within one laboratory. The formal statistical treatment of the data is outlined below.

Treatment of the results

Demonstration of attainment of equilibrium

50. The logarithm of the ratio of the concentration of the test compound in 1-octanol and water (log ($C_O / C_W$)) is calculated for each sampling time. Achievement of chemical equilibrium is demonstrated by plotting this ratio against time. A plateau in this plot that is based on at least four consecutive time points indicates that equilibrium has been attained, and that the compound is truly dissolved in 1-octanol. If not, the test needs to be continued until four successive time points yield a slope that is not significantly different from 0 at a p-level of 0.05, indicating that log $C_O / C_W$ is independent of time.
Log \( P_{OW} \)-calculation

51§3. The value of log \( P_{OW} \) of the experimental unit is calculated as the weighted average value of log \( C_o/C_w \) for the part of the curve of log \( C_o/C_w \) vs. time, for which equilibrium has been demonstrated. The weighted average is calculated by weighting the data with the inverse of the variance so that the influence of the data on the final result is inversely proportional to the uncertainty in the data.

Average log \( P_{OW} \)

52§4. The average value of log \( P_{OW} \) of different experimental units is calculated as the average of the results of the individual experimental units weighted with their respective variances.

The calculation is performed as follows:

\[
\log P_{OW, Av} = \frac{\sum w_i \times \log P_{OW,i}}{\sum w_i}
\]

where

- \( \log P_{OW,i} \) = the log \( P_{OW} \) value of the individual experimental unit \( i \);  
- \( \log P_{OW, Av} \) = the weighted average value of the individual log \( P_{OW} \) determinations;  
- \( w_i \) = the statistical weight assigned to the log \( P_{OW} \) value of the experimental unit \( i \) determination.

The reciprocal of the variance of log \( P_{OW,i} \) is employed as \( w_i = \text{var} (\log P_{OW,i})^{-1} \).

53§5. The error of the average of log \( P_{OW} \) is estimated as the repeatability of log \( C_o/C_w \) determined during the equilibrium phase in the individual experimental units. It is expressed as the weighted standard deviation of log \( P_{OW, Av} \) (\( \sigma_{\log P_{OW, Av}} \)) which in turn is a measure of the error associated with log \( P_{OW, Av} \). The weighted standard deviation can be computed from the weighted variance (\( \text{var}_{\log P_{OW, Av}} \)) as follows:

\[
\text{var}_{\log P_{OW, Av}} = \frac{\sum w_i \times (\log P_{OW,i} - \log P_{OW, Av})^2}{\sum w_i \times (\text{n}-1)}
\]

\[
\sigma_{\log P_{OW, Av}} = \left( \text{var}_{\log P_{OW, Av}} \right)^{0.5} \times \chi^2
\]

The symbol \( n \) stands for the number of experimental units.

Test Report

54§6. The test report should include the following information:

Test substance:

- common name, chemical name, CAS number, structural formula (indicating position of label when radiolabelled substance is used) and relevant physical-chemical properties (see paragraph 17);
- purity (impurities) of test substance;
- label purity of labelled chemicals and molar activity (where appropriate);
- the preliminary estimate of log \( P_{ow} \), as well as the method used to derive the value.

Test conditions:

- dates of the performance of the studies;
- temperature during the experiment, stirring rate (geometry of the test vessel);
- volumes of 1-octanol and water at the beginning of the test;
- volumes of withdrawn 1-octanol and water samples;
- volumes of 1-octanol and water remaining in the test vessels;
- description of the test vessels and stirring conditions, (geometry of the stirring bar and of the test vessel, vortex height in mm, and when available: stirring rate) used;
- analytical methods used to determine the test substance and the method limit of quantification;
- sampling times;
- number of replicates.

Results:

- repeatability and sensitivity of the analytical methods used;
- determined concentrations of the test substance in 1-octanol and water as a function of time;
- demonstration of mass balance;
- temperature and standard deviation or the range of temperature during the experiment;
- the regression of concentration ratio against time;
- the average value log \(P_{ow, A_{V}}\) and its standard error;
- discussion and interpretation of the results;
- examples of raw data figures of representative analysis (all raw data have to be stored in accordance with GLP standards), including recoveries of surrogates, and the number of levels used in the calibration (along with the criteria for the correlation coefficient of the calibration curve), and results of QA/QC;
- when available: validation report of the assay procedure (to be indicated among references).

LITERATURE


Annex

An example of glass-jacketed test vessel for the slow-stirring experiment for determination of $P_{OW}$.

Example of a test vessel for the slow-stirring method.