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Determining the water solubility of difficult-to-test substances: A tutorial review

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Abstract

Water solubility is one of the most important and frequently used physical-chemical properties of chemicals. It is crucial within several industrial sectors, in research and in the regulatory sector e.g. for the risk and hazard assessment of chemicals. The most recent OECD guideline (Test No. 105) for measuring solubility is from 1995 and limited to mono-constituent, stable and non-volatile substances. This OECD guideline and the described methods are not suited for several groups of difficult-to-test substances, such as highly hydrophobic chemicals, volatile chemicals, surfactants and mixtures. The aim of this paper is to review solubility measurement methods for difficult-to-test substances on a technical, analytical and scientific level. Methods to handle highly hydrophobic chemicals and volatile chemicals, and methods to rapidly saturate water with fast degrading chemicals are reviewed. A decision tree is presented outlining the preferred choice of method for each chemical group. This review also includes measurement methods for critical micelle concentrations that set the upper concentration limit for freely dissolved surfactants. Finally, concepts and strategies to measure solubility

parameters for mixtures, including multi-constituent substances and chemical substances of unknown or variable composition, are discussed.

Keywords: Water solubility, slow stir, passive dosing, volatile chemicals, hydrophobic chemicals

Abbreviations: UVCB: Chemical Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials; OECD: Organization for Economic Cooperation and Development; QSAR: Quantitative Structure-Activity Relationship; NAPL: non-aqueous phase liquid

1 Introduction

Water solubility can be defined as “the saturation mass concentration of the substance in water at a given temperature” [1]. Water solubility is one of the key physical-chemical properties of organic chemicals in environmental chemistry, analytical chemistry, environmental toxicology and many other fields of research and testing. The water solubility sets the upper limit for freely dissolved concentrations in aqueous solutions and thus for the exposure of aquatic organisms to chemicals in toxicity testing and the environment [2]. The water solubility sets the maximum concentration gradient that drives the diffusive uptake and bioconcentration in aquatic organisms [3]. While bound forms of the chemical can also contribute to the mass transfer and uptake kinetics of hydrophobic chemicals under dynamic conditions [4,5], the dissolved concentration, which is limited by the water solubility, is still the main governing parameter. Solubility is also important for the human uptake of chemicals since solubility can limit the uptake of drugs from the digestive tract to the blood. It is therefore an essential parameter in discovery and development of oral drugs [6,7].

The aqueous solubility is critical for the mobility, distribution and behavior of organic chemicals within experiments, analytical systems and our environment. Water solubility is for instance closely linked to the release and partition processes determining the transport and distribution of chemicals in the environment. Water solubility is therefore also a fundamental parameter in the risk assessment of chemicals, where it is used as input to exposure and fate models [8].

A number of models have been developed for estimating rather than measuring water solubility [9–15]. The uncertainty of such prediction models is currently approaching the uncertainty found in experimental and analytical solubility determinations [10,14]. Such prediction models are based on and calibrated with experimental data, and they therefore rely on sound experimental methods, accurate and precise analytical methods and carefully measured solubility values for a sufficient number of chemicals. It is essential to note

that the models are limited to the chemical domain included when constructing and calibrating the models, and therefore it is important that experimental methods are suited for the solubility determinations within a very wide chemical space.

Solubility experiments are conceptually quite simple and consist typically of two steps: (1) preparation of saturated water and (2) measurement of the chemical concentration in the saturated water. The OECD guideline No. 105 for solubility testing dates back to 1995 and is limited to mono-constituent, stable and non-volatile substances [1]. Fortunately, laboratory and analytical techniques have greatly improved during the last decades. Some of the new technical and analytical techniques may provide solutions to the limitations and challenges of the traditional methods.

The aim of this paper is therefore to review the current status regarding solubility testing and measurements of difficult-to-test substances (chemicals with low water solubility, volatile chemicals, fast degrading chemicals, surfactants, and multi-constituent substances [2]) on a technical, analytical and scientific level. The section on technical challenges deals with the preparation of saturated water, i.e. the first step of solubility experiments. This part focuses on methods to avoid micro-droplets or crystals of pure chemical in the water phase, on volatile chemicals and on reducing the saturation time in order to handle chemicals which are not stable in water over longer time-frames. The section on analytical challenges deals with the measurement of highly hydrophobic chemicals in saturated water, i.e. the second step of a solubility experiment. The section on scientific challenges focuses on the concept of solubility for surfactants and mixtures including nonpolar hydrophobic UVCBs.

2 Determining water solubility

Two basic principles can be used when determining water solubility experimentally. One group of methods determines solubility by establishing equilibrium between an excess amount of test chemical and its dissolved form in pure water. These methods have been termed 'variable composition' [16] 'excess solid' [17] or 'thermodynamic' methods [6,18,19]. Another group of methods are termed 'apparent', 'excess solvent' or 'kinetic' solubility methods [6]. They determine solubility using a fixed amount of test chemical and varying factors such as temperature or volume of water to reach the solubility point or 'cloud point' from undersaturation, oversaturation or a combination of these [6,16]. By doing this, the solubility of the fastest precipitating crystal structure is measured, and these methods are therefore likely to overpredict the thermodynamic solubility [6,18]. They are also much more sensitive to impurities [17] and more prone to supersaturation artifacts than thermodynamic methods [20,21]. They are widely used in drug discovery because they can be set up as high-throughput methods requiring a minimal amount of solute [6], however they cannot substitute for careful thermodynamic solubility determinations [7]. The focus of this review will therefore be on the thermodynamic methods.

Important factors to ensure high quality solubility determinations include high purity of the test chemical, high purity of water, stability of the test chemical during equilibration, temperature control of the experiment, sufficient agitation, sufficient time for the water to reach saturation, and complete separation of the soluble fraction from the pure phase [16,22].

The current OECD guideline for water solubility testing recommends two approaches for saturating water by equilibrium partitioning with test substance; the shake-flask method for chemicals with a solubility above 0.01 g L⁻¹ and the column elution method for chemicals with solubilities below this limit [1]. The shake-flask method consists of mixing solute and water at the desired temperature until equilibrium is reached (see Figure 1), and

then separating the non-dissolved test substance from the water phase by either filtration or temperature controlled centrifugation [1]. The column elution method consists of pumping water through a column containing an inert support material (e.g. glass beads) coated with the solid chemical (Figure 1). The flow rate can be adjusted to ensure full saturation of the water passing through the column or the water can be recirculated through the column until equilibrium is reached, as defined by five successive samples that do not differ by more than $\pm 30\%$ in concentration in a random fashion [1]. For both methods, the concentration in the water is subsequently measured with an appropriate analytical technique [1]. An adaptation of this method, the generator column method [23], is included in the United States Environmental Protection Agency water solubility guidelines [24]. It is closely related to the column elution method as it uses the same saturation principle. However, the generator column method includes automated extraction and analytical procedures for the water exiting the saturation column [23,25,26].

The ability to experimentally establish equilibrium between phases is important not only for solubility determinations. Experiments to determine the octanol-water partition ratio of organic chemical requires equilibrium of a chemical between a water phase and an octanol phase. For this purpose, a slow-stir method has been developed and adopted into the OECD guidelines [27–29]. The method has subsequently been used for solubility determinations by various groups [30–33]. It consists of saturating water with a low density liquid test chemical through carefully controlled magnetic slow stirring (no vortex) in order to avoid emulsion of the chemical and water (Figure 1). The equilibrated water is collected from the bottom of the test system for analysis. The same principles can be used for solubility determinations of low density liquids, when the magnetic stirrer at the bottom is replaced by another stirring mechanism operating from the top of the bottle. However no examples of this have been found in the literature.

In aquatic toxicity testing, a range of passive dosing methods has been developed to control aqueous concentration of hydrophobic organics at their solubility limit and at defined concentrations below solubility [34–39]. Passive dosing applies a polymer pre-loaded with the test substance for establishing and controlling constant aqueous concentrations by equilibrium partitioning between polymer and water [40]. Systems are designed with dimensions (polymer/water/headspace ratios) allowing the polymer to dominate the partitioning of the test chemical in order to control the freely dissolved test chemical or chemical activity in the system (Figure 1). The polymer needs to be loaded to the saturation level when applying passive dosing for solubility determinations. For solids, two different principles have been used: (1) Saturated methanol suspensions with solid hydrophobic chemicals were prepared and then used to saturate the silicone polymer. After cleaning of the polymer to remove methanol and remaining crystals, water was added and saturated by equilibrium partitioning with the saturated silicone [35]. (2) Crystals of solid hydrophobic chemicals have been cast into silicone polymer and after curing and cleaning the silicone, water was added and saturated by partitioning from the loaded polymer [41]. For liquid test chemicals, the silicone polymer can be saturated by direct immersion of the polymer in the pure liquid chemical. After carefully cleaning the polymer surface, the saturated polymer can be used as partitioning donor to saturate water [42]. Volatile liquid substances can even by themselves serve as partitioning donor to saturate water. Such a headspace passive dosing method has recently been developed for toxicity testing, where water was saturated directly from the pure liquid through the headspace [43] (Figure 1).

Methods have also been developed for solubility testing of volatile liquid chemicals. Sanemasa et al. designed a saturated vapor method [44], which was later simplified by Dohányosová et al. [45]. The water was saturated by bubbling an inert gas through the liquid test substance and then through the water (Figure 1).

3 Technical challenges

3.1 Ensuring full separation of undissolved and dissolved chemical

A crucial aspect of determining water solubility is the ability to avoid or remove non-dissolved chemical from the solution after the equilibration phase. This means ensuring that there is no particulate phase, microcrystals, emulsions or micro-droplets in the water sample that is extracted for analysis. Loftsson [19] found that chemicals with low solubility are more prone to overestimation of solubility due to formation of nano-sized aggregates than chemicals with high solubility. This challenge therefore mainly relates to hydrophobic chemicals. The practical challenges are slightly different for chemicals which are in the solid state than for chemicals which are in the liquid state at the desired temperature. However, three main approaches have been used in order to overcome the challenges (1) efficient removal of microcrystals/droplets after equilibration, (2) reducing agitation in order to eliminate formation of microcrystals/droplets and (3) avoiding direct contact between the pure chemical and the water during equilibration.

The presence or absence of micro and nano-sized droplets in the saturated water can, if necessary, be investigated using methods such as laser particle counting [46], dynamic light scattering, or other light scattering methods used for the determination of the critical micelle concentration of surfactants (see section 5.1)

3.1.1 Solid hydrophobic chemicals.

The shake-flask method is prone to formation of microcrystals because of the high agitation during equilibration, and for this reason the shake flask method is not recommended for hydrophobic chemicals [1]. Methods to improve efficiency in removal of microcrystals include dialysis and ultrafiltration, which have been used when measuring the solubility of nanomaterials [47]. However, even these are not considered sufficiently reliable to fully remove nano-sized particles from solution [47].

The generator column and column elution methods, based on passing water through a column with a high surface area support materials coated with the test chemical, were developed in order to reduce the formation of microcrystals in the saturated solutions by reducing the agitation during equilibration of solid chemicals with water [23]. Some studies have observed higher initial concentrations in the water produced by the generator column, likely due to loose crystals detaching from the column [41,48]. The concentrations, however, decreased with recirculation of the water through the column, during which, the detached crystals were most likely retained in the column. The generator column method was found to produce lower and more reliable solubilities than the shake-flask method for the hydrophobic polycyclic aromatic hydrocarbons (PAHs) because of the reduction in formation of microcrystals [48].

Methods have also been designed to avoid contact between the pure chemical crystals and the water [41,49,50]. Akiyoshi et al. proposed a vapor generator method using saturated solvent vapor to transfer the chemical to the water [49]. However, the solubility of the substance can change in the presence of another solvent, so this method may bias the solubility determination [41]. The use of water vapors to transfer the chemical was also tested, but the equilibration times increased substantially [50]. The method has not gained considerable use. Considering that most solid state hydrophobic chemicals are more lipophilic than volatile, it seems more promising to use passive dosing methods. Kwon and Kwon [41] casted an excess amount of crystals (five PAHs) into silicone (polydimethylsiloxane, PDMS) in order to saturate the silicone while leaving a crystal layer beneath the PDMS at the bottom of the vial. Water was then equilibrated with crystals through the saturated PDMS producing similar results as the column elution method. This way they reduced the direct contact between the water and the crystals, decreasing the risk of crystals detaching from the column. This method is thus promising for solid lipophilic chemicals.

3.1.2 Liquid hydrophobic chemicals.

The vigorous agitation used in the shake flask method is challenging for liquid hydrophobic chemicals as well as for solid hydrophobic chemicals. Peake and Hodgson [51] found that shaking water and alkanes produced concentrations above water solubility which depended on the subsequent filtration pore size. This could be due to the formation of micelles in solution [51].

The generator column method [26] may reduce the formation of micro-droplets in the water because it reduces the agitation compared to the shake-flask method [52]. However, this method is not reliable for liquid test chemicals as there is a possibility for the liquid test chemical to slough off the support material during equilibration [1]. In an extreme effort to avoid formation of micro-droplets, Coates et al. equilibrated alkanes in a static system where diffusion was the only transfer process between the water and the liquid solute. They found equilibration times in the range of 90 days [53]. These high equilibration times are of course not practical, and a compromise is to use the slow-stirring method (see Figure 1). The slow stir method has been preferred to avoid emulsion problems when determining solubilities for hydrophobic liquid chemicals such as phthalates [30], hydrocarbons [32,33] and alcohols and diesters [31]. The slow stir method has been argued to be more accurate than other methods: The solubility of di(2-ethylhexyl)adipate determined by the slow stir method was consistent with values predicted from molecular structure (using the physicochemical calculator SPARC) but over two orders of magnitude lower than the shake-flask method [54]. Furthermore, molecular dynamic simulations used to estimate water solubilities of n-alkanes agreed well with results using the slow-stir method, but not with results from the shake-flask method [11]. The disadvantage of the slow stir method is the long equilibration times. Equilibration times of one-two weeks [31,32,54] or three days [33] have been reported in different studies. However, the slow stir method is a robust method to determine solubility of liquid hydrophobic chemicals.

For liquid chemicals, methods have also been proposed to separate the pure chemical from the water phase in order to avoid formation of micro droplets. For semi-volatile and volatile chemicals, equilibration through the headspace has been used. Sutton and Calder [55] used half-filled 1 l flasks with an insert containing liquid alkylbenzenes. The alkylbenzenes equilibrated with the water through the headspace within 35 hours [55]. Equilibration through headspace was also evaluated by Tolls et al. [33]. Without details of the test system dimensions, they reported equilibration times of 3 weeks for decane, making them decide to use the slow-stir method instead. Trac et al. [43] recently described a headspace passive dosing setup for toxicity testing at saturation with dimensions allowing equilibrium of water from a pure phase liquid within 24 hours (Figure 1). Compared to the prior test set-ups, the surface area to volume ratio of the water phase was increased to increase transfer kinetics. The headspace passive dosing systems require tight temperature control in order to avoid condensation of the pure chemical in the test system. Apart from this the test systems are quite simple.

Passive dosing of liquid chemicals from a silicone polymer is another possibility to separate the pure phase chemical from the water. A passive dosing method, where silicone was saturated by immersion in the pure liquid chemical and subsequently used to saturate an aqueous medium, worked well with silicone o-rings [42]. Another method is to dose through liquid filled tubes. This method has been demonstrated using two oils and stirring of either the water phase [56] or stirring of the oil filled silicone tubing tied to the stir bar at 300 rpm [57]. The method is however vulnerable to production of micro-emulsions [42] and leakage from the ends of the tubing [46]. From current experiences, special attention is necessary regarding careful closing of liquid filled tubes, careful physical cleaning of saturated polymers and appropriate shaking/stirring of the systems in order to avoid emulsion formation if using passive dosing of liquid chemicals at saturation.

3.2 Reducing equilibration times

The strongest driver for developing fast methods to determine solubility is the practical aspect which is closely linked to costs. Miniaturized methods for thermodynamic solubility determinations have therefore been developed for high-throughput in drug discovery [6]. Since 87% of marketed drugs in a 1997 study had turbidimetric solubilities above 65 mg L^{-1} [7], the focus of solubility testing in drug discovery is the mg L^{-1} range [7,58], and the shake flask method is used as a basis for high-throughput methods. A review by Alsenz and Kansy [6] describes automation developed for powder dispensing in microtiter plates, automation for dispensing of solvents with low or high viscosity, mixing in small systems, filtration of the solutes, pH determinations, and characterization of solid state properties in high throughput screening. One example of an automated setup, is a 2 mL test system (Whatman UniPrep filter chamber) equilibrated for 24 hours after which a plunger with a filtration membrane is pressed into the chamber to filter out the crystals from the medium before HPLC measurements [20,59]. In this system, equilibration kinetics are not determined, and the full saturation therefore not proven.

Solid chemicals may have different crystalline forms, where metastable forms have a higher solubility than the stable form. While solubility can be measured for any crystalline form as long as the structure do not change during the measurement, it can be important to characterize the solid state properties after the measurements [6]. In some cases it is crucial to determine the solubility of the most stable polymorph [6], which can be produced by solvent mediated polymorphic transformation. The choice of solvent for this is important for the transformation rate [60]. A modified shake-flask method was adopted by Loftsson and Hreinsdottir [61] in order to shorten equilibration times for solid chemicals while measuring the solubility of the most stable polymorph. An excess amount of solute was added to water, which was supersaturated by heating to 121°C for 20 min. followed by cooling. Then the water was seeded with the stable crystalline compound and equilibrated

for 3-7 days during which the supersaturated material precipitated. The seeding ensured that the most stable crystalline form of the chemical was produced during precipitation.

A rapid method, called CheqSol, has also been developed for establishing equilibrium in solubility determinations of ionizable compounds. The CheqSol approach is an automated titration method where pH is adjusted in small steps and the direction of precipitation/dissolution determined by the direction of the subsequent pH change. The point of equilibrium is thus found by changing the concentration of the neutral form of the solute by the pH change [21]. The method works down to 1 mg L^{-1} , and is therefore not useful for hydrophobic chemicals.

The high-throughput methods described above were developed in order to save time in the laboratory, and not in order to expand solubility determination methods towards difficult-to-test substances. These methods are therefore not designed to be appropriate for hydrophobic chemicals, volatile chemicals or fast degrading chemicals.

3.2.1 Fast degrading chemicals

Some may argue that a solubility determination has limited environmental relevance for fast degrading chemicals. However, there are scenarios where the volume of chemical present would still warrant a thorough hazard assessment. Furthermore, since solubility is a fundamental physical-chemical property, it is part of the characterization of the chemical even though it may in some cases not be important for the fate of the chemical.

Test chemicals can be subject to abiotic degradation processes during solubility testing such as photolysis, oxidation and hydrolysis. Most solubility test systems can be adapted to avoid photolysis during equilibration. Amber glass bottles can be used for shake flask experiments, the test system can be shielded from light by the

temperature controlling water jacket around the system or by aluminum foil, and in the case of slow stir methods, bottles can simply be placed in incubators [32] in the dark.

Chemicals prone to oxidation are easier to handle in solubility testing compared to toxicity testing [62], because oxygen saturated water is not necessary for the successful determination of water solubility. Test water should then be kept anaerobic by flushing with nitrogen gas, and tests conducted using a reduced or eliminated headspace as has been done when testing volatile chemicals with the slow stir method [32].

Chemicals for which hydrolysis can occur are more problematic. As already mentioned reaching equilibrium between pure substances and water may require long equilibration times. For fast degrading chemicals it is therefore crucial to shorten saturation times in order to be able to determine the solubility of these chemicals with a reasonable accuracy.

Two methods have been proposed where equilibrium of the chemical with the water can be obtained within minutes. The first method with potential for very fast equilibration kinetics is the passive dosing method. Equilibration kinetics in passive dosing increases with increasing donor surface area to medium volume ratio and also with increasing degree of stirring of the system. It is therefore possible to optimize passive dosing systems for fast equilibration. The system dimensions in the study by Stibany et al. [42], were not optimized for very fast equilibration times, and 11 hours were needed to establish equilibrium with algal medium containing 20 % methanol. Equilibration times less than 10 minutes have been demonstrated for passive dosing of hydrocarbons to water in an optimized design [63,64]. The concentrations in this study were below solubility, but very similar equilibration times are expected also for equilibration at the solubility limit. For solid and unstable chemicals, an accelerated passive dosing system seems to be the best option, as this method worked well at the solubility point [41].

For liquid test chemicals, initial experiments have revealed that passive dosing at the solubility limit can be vulnerable to the condensation of chemical and the subsequent droplet formation. Loading the silicone to 90% of the saturation level resulted in more reproducible results (unpublished data). The most efficient and practical way to avoid micro droplets would then be to load the polymer just below saturation. Running such experiments at several loadings below saturation (e.g. 50, 70, 90 and 100 %) might be the best way to approach the solubility level from below, while generating the necessary measurements to detect and discern any micro droplet formation artefacts.

The second method with potential for very fast equilibration kinetics is the saturated vapor method for liquid test chemicals. This method ensures a high agitation while keeping the solute phase and the water phase separate (avoiding droplet formation). The equilibrium time was 3 minutes [44]. However, also this method is vulnerable to condensation of the chemical in the water reservoir. Therefore, it was better at producing reproducible concentrations when the temperature of the solute reservoir was lower than that of the water i.e. when the water was slightly under-saturated, and poor precision was observed when the temperatures were equal, exactly at the solubility point [44].

Even though there are drawbacks for the two methods for liquid chemicals (saturated vapor and passive dosing), the methods provide sufficiently short equilibration times for fast degrading chemicals.

4 Analytical challenges

As stated in the introduction, solubility experiments consist of (1) preparation of saturated water and (2) measurement of the chemical concentration in the saturated water. As with the first step, the measurement of chemicals is subject to challenges dependent on the test substance.

4.1 Volatile chemicals

A high volatility can be used as an advantage when testing the solubility of chemicals, but it can also be a challenge. For volatile chemicals, headspace passive dosing methods can be used to saturate water via the headspace, while avoiding a direct contact between test substance and water and thus avoiding non-dissolved test chemical entering the water phase (Figure 1). However, volatile chemicals are also more prone to losses during extraction and analysis of the water sample after equilibration. It is therefore important to minimize or even eliminate the steps between equilibration and analysis, which can be done by a close alignment between the experiment and the instrumental analysis. Much can be done with careful handling of the water, using gas-tight syringes, avoiding headspace etc. Letinski et al. (2016) successfully determined solubility for highly volatile hydrocarbons by using gas-tight syringes for sampling the saturated water, adding the samples to headspace vials and performing analysis using headspace solid phase micro-extraction coupled to GC-MS without storage. Tolls et al. [33] also used gas-tight syringes and a closed purge and trap system and were careful not to lose chemicals by evaporation.

Multiple headspace extraction gas chromatography is a method specifically developed for volatile organic chemicals, which aligns the solubility experiment with the analysis [65]. Technically it is a kinetic method, where water with an excess amount of solute is used, and then multiple headspace extractions remove solute from the headspace while measuring the concentration in the headspace using GC analysis. As long as there is excess pure phase chemical in the water phase, the concentration in the headspace will be buffered by the

pure phase chemical, however, when there is not any more pure phase left in the system, headspace concentrations will decrease fast [65]. Care should be taken with this method to ensure that agitation and equilibration time is sufficient and that sorptive losses to septum and other surfaces or impurities do not interfere with the measurements.

4.2 Highly hydrophobic chemicals

The aqueous solubility of highly hydrophobic chemicals is very low. One of the challenges for this group of chemicals is therefore related to analytical detection of very low concentrations [66]. Developments in analytical chemistry within the last 20 years have ensured that concentrations in the ng L^{-1} range can be measured for almost all organic chemicals using liquid-liquid extraction or solid phase extraction and GC-MS or LC-MS. Tolls et al. [33] used for instance headspace SPME on 25-500 μL samples (quantitative extraction) for alkanes ($\mu\text{g L}^{-1}$ range) and purge and trap analysis for alkanes with even lower solubilities (ng L^{-1} range) where the SPME method did not provide sufficient sensitivity. Lower concentrations, in the pg L^{-1} range or lower, is however still challenging to quantify.

Careful sample handling is particularly important for highly hydrophobic chemicals in water samples. Avoiding pure phase micro-droplets and ensuring that there is no dissolved organic carbon or dust contamination of the water, which would lead to overestimation of solubilities, can be quite challenging, and is preferably handled during the equilibration step. However, highly hydrophobic chemicals have a high potential for sorptive losses during the analytical work, which then can lead to an underestimation of the solubility. The immediate addition of non-polar solvents to aqueous samples for liquid-liquid extraction before GC measurements as well as the immediate addition of more polar solvents to aqueous samples before HPLC measurements can reduce such losses. If available, ^{13}C -labeled substances can be added via the solvent, which then can be used to correct for losses and incomplete extractions.

Background contamination of the test chemical can be of crucial importance for highly hydrophobic chemicals, especially when solvents are used for extraction. During the equilibration step, the pure phase chemical is handled in the laboratory. In the subsequent step, concentrations >10 orders of magnitude lower have to be handled without any contamination. High care must therefore be taken regarding the solvents and equipment used in sample handling steps to avoid contamination with the test chemical in order to avoid overestimation of the solubility. A clear separation between equipment used in the two phases of the solubility measurements is recommended. Background contamination stemming from a wide use of the test chemical in equipment used in the laboratory or its surroundings is harder or may be even impossible to get rid of.

5 Scientific challenges

5.1 Surfactants

Surface active compounds, or surfactants, are molecules with a polar head and a nonpolar tail. Technically these compounds are highly miscible with water. However, above a certain concentration in water, the critical micelle concentration (CMC), the surfactants cluster into micelles, and are thus not freely dissolved anymore. This can be viewed as equivalent to the formation of micro-droplets or emulsions of hydrophobic chemicals in water. The difference is that the formation of the micelles cannot be avoided by eliminating agitation, and the micelles are uniformly distributed within the water phase. This also means that even given quiescent conditions and long time-frames, the micelles will not separate from the water phase to form a pure phase at the top or bottom of the water.

Since the critical micelle concentration sets the upper limit for freely dissolved molecules, it is sometimes used as a surrogate for the solubility of surfactants [67]. The OECD guidance document recommends that exposure concentration in toxicity testing are expressed as nominal concentrations but that toxic effect concentrations are compared to the dispersibility limit or the critical micelle concentration instead of the solubility limit [62].

Most methods for determination of the CMC are indirect methods. This means that the equilibrium concentration of the freely dissolved molecules in water is not measured. Instead, physical or chemical properties are measured over a concentration range, and the CMC is inferred from a change in their trend. The most common physical methods are surface tension [68–70], electrical conductivity [71,72] and spectroscopic methods [73].

The surface tension method works by the principle that below the CMC the surface tension decreases as surfactant concentration increases, whereas above the CMC this increase levels off since additional surfactant molecules form micelles. However, surface tension methods have the drawback that the detected changes do not refer directly to the bulk phase of the solution but to phenomena at the surface. Data may therefore be challenging to interpret, and in some cases erroneous results are found. These errors can occur if the surface interface is saturated with molecules at concentrations below the CMC [68] or an efficient packing structure of the chemicals at the interface leads to increased capacity of the interface for surfactant molecules above the CMC [74].

The electrical conductivity method consists of measuring the specific conductivity of surfactant solutions and is limited to ionic surfactants [75]. In most cases a sharp break will be seen in the conductivity at the CMC, and the data are easy to interpret. In cases where a weak curvature is seen in the region around the CMC, a number of different mathematical procedures can be used for the analysis of the experimental data [75,76] or dielectric constants can be measured instead [77].

If surfactants have aromatic fragments in their structure, the CMC can be measured by non-invasive spectroscopic methods, where the CMC is measured in a solution containing only the surfactant and water using absorbance or fluorescence spectroscopy [78–80]. Otherwise, invasive spectroscopic methods can be

used, where a tracer is added to the solutions before measurement. Examples of tracers used for spectroscopic methods are summarized in Supplementary material S1.

Several other methods have been developed for CMC determinations. Non-invasive physical methods include capillary electrophoresis [81–83], speed of sound measurements [84,85], resonance Rayleigh scattering [86], static and dynamic light scattering [74,87,88], potentiometry [89], nuclear magnetic resonance, NMR [90,91], diffusion ordered spectroscopy NMR [92,93], ultrasonic spectroscopy [94], fiber-optical measurements [95], induction current [96], chemiluminescence [97] and chiroptical spectroscopic methods [98].

Partitioning of hydrophobic chemicals to the micelles can also be used to determine CMC by measuring the solubility enhancement of solvent solutions [64,99], and recently a method was suggested where the partitioning of toluene between headspace and solution was used for automated CMC measurements [100].

All of the above mentioned methods are vulnerable to loss processes because nominal concentrations are used to infer the CMC and the concentrations of the surfactants in solution are not measured. This would mainly affect measurements of surfactants with a low CMC and high affinity for partitioning to interfaces. A method where the dissolved concentrations in the water is measured to determine solubility of surfactants was described by Haftka et al. [67]. They used solid phase microextraction fibers to measure the concentration of the freely dissolved surfactant in order to determine the CMC for a range of neutral, zwitterionic, anionic and cationic surfactants. The method is limited to surfactants with CMCs below the aqueous concentration corresponding to the binding capacity of the fiber [67]. Ultrafiltration combined with centrifugation has also been used to separate the freely dissolved molecules from the micelles for CMC measurements [101–103].

5.2 Multi-constituent substances

Water solubility is first of all an intrinsic property of pure chemicals. However, use and emissions of chemicals often occur as mixtures. For mixtures with one dominant component and a minor impurity, solubility

determinations should target the solubility of the main component, which requires dedicated analytical techniques to separate and quantify the main component in the presence of the impurity. The next level of complexity is multi-constituent substances, which include mixtures of chemicals, congeners, isomers and enantiomers. Saturated aqueous solutions of multi-constituent substances do not provide a real “solubility” in the strict sense. However, they are still highly relevant for the fate, exposure and toxicity of multi-constituents substances.

The simplest mixtures contain a limited number of pure chemicals. The concentrations of the mixture components in water equilibrated with an excess amount of such mixtures depend on whether the chemicals are in the liquid state or solid state and how the components in the mixture interact (within the mixture and in the water) [104].

If the activity coefficients of all *i*th components in a mixture, $\gamma_{i,mix}$, approximates 1, then the mixture is called an ideal mixture [104]. This means that the intermolecular interactions between a molecule of the *i*th component and other components in the mixture are approximately similar to the intermolecular interactions between two molecules of the *i*th component [105].

When the mole fraction does not change much during equilibration in the test system, e.g. at high loadings, the following relationships applies for dissolved concentrations of mixture components. For ideal liquid mixtures, the equilibrium water concentration of the *i*th component, C_i , is related to the water solubility of the pure form of the *i*th component, S_i , by the mole fraction of the component in the mixture, $x_{i,mix}$ as shown in equation 1 (Raoult's law) [104,105].

$$(1) C_i = x_{i,mix} \cdot S_i$$

For ideal solid mixtures, the equilibrium water concentration of each component is however independent of the composition of the mixture (equation 2) [104,106,107].

$$(2) C_i = S_i$$

Ideal mixtures may also comprise of a mix of chemicals, which are liquid and solid in their pure form. If the liquid components dissolve the solid components, the mixture will behave as a liquid mixture, and the equilibrium concentration depend on the mole fraction of each component in the mixture. However, for the solid components, the equilibrium will not be a fraction of the solubility of the solid pure form, but of the subcooled liquid solubility of the chemical, which is higher than the solid solubility (equation 3) [104].

$$(3) C_i = x_{i,mix} \cdot S_{i,subcooled\ liquid}$$

If the amount of solids in the mixture exceeds what can be dissolved in the liquids, they will crystalize and behave according to equation 2 [104].

The saturation concentrations in water from non-ideal mixtures are not as straight-forward to predict. Mixtures of hydrophobic and hydrophilic components are non-ideal mixtures. When these mixtures are equilibrated with water, two factors affect the equilibrium concentrations: 1) A significant amount of water can partition into the mixture and thereby increase the activity coefficient of the hydrophobic chemical in the mixture, and 2) a significant amount of the hydrophilic components can partition into the water and thereby reduce the activity coefficient of hydrophobic component in the water, the co-solvent effect. The equilibrium concentration of the hydrophobic component will then be increased compared to the expectations from equation (1). The activity coefficients of the component in the mixture, $\gamma_{i,mix}$, and in the water $\gamma_{i,water}$, can be predicted from group contribution structure-property relationships (the UNIFAC method [108], UNIFAC

appears to have comparable performance relative to other physicochemical property models [109]), and the equilibrium concentration in water can be described by equation (4),

$$(4) \quad C_i = \frac{x_{i,mix} \gamma_{i,mix} \gamma_{i,water}^p \cdot S_i}{\gamma_{i,water}}$$

where $\gamma_{i,water}^p$, is the activity coefficient in the water of the pure chemical [104].

In non-ideal solid mixtures, constituents interact and e.g. form solid solution [106] or eutectic structures [110]. For interacting solid mixtures, the composition of the solid solution will influence the solubility of the components [106,110], and the resulting equilibrium concentration in water will most often be somewhere in between what would be expected from ideal liquids (equation 3) and ideal solids (equation 2).

Special attention is needed for analytical determination of the solubility of multi-constituent substances. In principle, the methods for solubility determination of single chemicals can still be used for multi-constituent substances. However, it is then crucial to use a sufficiently high mixture to water ratio to ensure that the mixture composition is not significantly changed during equilibration.

5.3 UVCBs

While it may be complicated to define and determine the “solubility” of multi-constituent substances of known compositions, an even more complicated matter is to characterize and predict saturated solutions of Chemical Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials (UVCB’s). In a specific mixture of known composition, the saturated concentration of each constituent can be predicted as described above. However, for UVCB’s, the composition is not fully known and variable. A meaningful parameter could then be the total dissolved concentration of the UVCB components in pure water in equilibrium, while ensuring that none of the components are depleted during equilibration. Two focus points are therefore especially important when characterizing saturated solutions of UVCB’s: 1) determination of

aqueous concentrations of mixtures which are not fully characterized and 2) ensuring a constant mixture composition during the equilibration (avoiding depletion of single constituents).

The water:mixture volume ratio necessary to avoid depletion of mixture components, depends on the partition ratio of the constituents between the mixture and the water. It can be estimated if the mixture-water partition ratio of the least hydrophobic constituent is known. For petroleum mixtures, the minimum octanol-water partition ratio of components in the mixture, $K_{ow,min}$, can be used to estimate this ratio if partitioning between the mixture and water phase is assumed to roughly follow octanol-water partitioning. If partitioning to headspace is also neglected, then based on mass balance calculations, a minimum water:mixture ratio, $R_{w:m,min}$, to ensure less than 5% depletion of the mixture can be found by $R_{w:m,min}=0.05*K_{ow,min}$. These rough mass balance calculations are in line with observations reported in the literature. Of eight investigated compounds in a diesel fuel (benzene, alkylbenzenes, and PAHs), only benzene was seen to deplete from the diesel (by 10%) when using a water:diesel ratio of 20 [111]. Shiu et al. [112] found that a water:oil ratio of 20:1 or 10:1 was sufficient to avoid depletion for three crude oils and a gasoline, however, for fuel oil no. 2, which contained a large fraction of pentane and lighter constituents, a low water:oil ratio of 5:1 was not even sufficient [112].

Saturated solutions of liquid UVCB's have been generated using the same approaches as for the pure liquid chemicals including shake flask [113], slow stir [111,112], generator column [114], passive dosing methods [56,57], and modeling [115].

Experimental methods to prepare saturated solutions of UVCB's face the same challenges as for pure substances regarding avoiding micro-droplets and emulsions of hydrophobic liquid chemicals and losses of volatile chemicals. The slow-stirring method is therefore often preferred for petroleum products [111,116]. Equilibration times of 1-2 days were seen in one study, but it was pointed out that for viscous UVCBs, transport within the complex mixture may in some cases slow down the transport of the solute to the water [111].

Saturated solutions of UVCB's are of special concern when evaluating the leaching potential from non-aqueous phase liquids (NAPLs) in soil. Mao et al. [114] investigated the saturated solutions of 13 mixtures of petroleum hydrocarbons using a column generator type of set-up, where petroleum spiked sand was added to a column and water pumped through to generate saturated solutions. Analysis was done using HPLC-GCxGC, in order to identify 79 hydrocarbon fractions. Indicator compounds and their solubility were assigned to each fraction, and Raoult's law used to calculate total saturated concentrations for each petroleum product, which corresponded within a factor of 3 to the experimental solubilities. Deviations were assigned to non-ideality of the chemicals in the mixtures and losses during sample extraction and analysis [114].

The total saturated concentration of UVCBs in water is a challenging subject because it is determined by the sum of solubilities of the individual constituents. The variable physicochemical properties of the constituents can result in variable fate of chemicals in test systems, and the environment. For example, volatile constituents will partition to air phases, soluble constituents will remain in water, and insoluble constituents will remain in the UVCB, and all of the constituents exist along a spectrum of volatility and solubility. UVCB concentration will also change over time because of the 'weathering' of the mixture, where the constituents of highest solubility and volatility are depleted from the mixture. Depletion of constituents over time (>20 d) was noted in oiled gravel studies resulting in time variable exposures [117,118].

In modeling approaches relevant descriptors of the UVCB mixture are crucial. A number of UVCB's such as gasolines, diesel fuels and coal tars have been shown to reveal near-ideal behavior [114,115,119–122]. These UVCB's can be characterized by block methods or structure oriented lumping methods, where constituents are lumped into groups based on their structure and/or size [123,124]. Representative water solubilities can then be assigned to each block based on relevant measurements or solubility models. For near-ideal mixtures, saturated concentrations in water for block components can be modeled using the equations for ideal mixtures

(Raoult's law) based on representative structures in the mixtures [46,125]. For polar components such as phenols, anilines and benzotriazoles in nonpolar fuels, descriptors for polar molecular interactions has to be included in the models in order to produce reliable solubility predictions [113]. Dissolution of constituents from a UVCB is generally controlled by the mole fraction of the constituents in the UVCB phase. However, volatile or soluble constituents can partition to the aqueous or air phase and alter the mole fraction of the parent oil. Therefore, a mass balance model is needed to account for the depletion. Application of a mole fraction-based mass balance solubility model has been successfully applied to petroleum substances [46,57,126]. This modeling framework can describe the solubility of constituents with a wide range of physicochemical properties, and also accounts for depletion of the test substance at low substance loadings. This approach is an extension of the hydrocarbon block method [127], which define a petroleum substances into blocks by chemical class and carbon number. The physicochemical properties are generally estimated by QSAR. However, advances in data analysis of GCxGC analytical results provide an opportunity to refine predictions of saturated concentrations by providing methods to assign properties to individual peaks instead of aggregated blocks.

6 Recommendations for solubility determination of difficult-to-test substances

Based on this literature review, the authors suggest a decision tree for solubility determinations of difficult-to-test chemicals as shown in Figure 2. As described in section 5.1, surfactants represent special challenges for solubility measurements, which are not covered in this decision tree. Readers are referred to section 5.1 for methods to determine CMC. Mixtures are also not included in Figure 2. When determining solubilities of mixtures, the same issues regarding stability, hydrophobicity and volatility apply as for pure chemicals. Therefore, analytical methods can be chosen based on the chemical properties of the major fraction of components in the mixtures. The decision tree includes criteria for categorization of chemicals in each group: hydrophilic/hydrophobic/very hydrophobic, solid/liquid, stable in water/not stable in water, and volatile/non-

volatile. The cutoff between the hydrophilic/hydrophobic group is based on the literature data presented by Ferguson et al. [11]. It showed that solubility data for alkanes of chain length up to heptane had little variability, data for octane to decane had some variability, and data for alkanes with a chain length longer than decane had high variability. These solubility data mainly stemmed from measurements using the shake flask method. Even though the current OECD guideline [1] recommends a solubility cut-off of 10 mg L⁻¹ for the shake-flask method, we therefore assess the method to be applicable down to solubilities of at least 1 mg L⁻¹. The expected solubility of the test chemical can be estimated prior to experiments using QSAR relationships [128] in order to select the appropriate test method with the decision tree. However, it should be noted that uncertainty in the order of a couple of magnitudes can be associated with such estimations [9]. Experimental Log *K*_{ow} values can be used instead of estimated solubility values. For chemicals with high molecular weight, solubility estimations are however more appropriate to use, since large chemicals tend to have lower solubilities in both octanol and water. Also, for chemicals with a high melting point the grouping between hydrophilic, hydrophobic and very hydrophobic will be somewhat different when based on Log *K*_{ow} compared to water solubility. The reason for this is that water solubility not only depends on the hydrophobicity (i.e. activity coefficient in water), but also on the enthalpy required to transfer the solid chemical to a liquid state (i.e. melting enthalpy directly related to melting point) [129].

Stability of the chemical relates primarily to degradation processes that are difficult to eliminate by controlling the oxygen, light or sterility of the test system. Hydrolysis is the most important degradation process that is hard to eliminate, but biological degradation can also be difficult to avoid when the testing requires prolonged equilibration times. No specific methods were found in the literature describing solubility testing of chemicals that hydrolyzes rapidly. However, acid and base catalyzed hydrolysis can often be reduced by adjusting to the pH at which the test substance is most stable. For the purpose of using the flowchart, an estimated half-life based on these non-controllable degradation processes would assist in choosing the appropriate testing

methodology. Hydrolysis half-lives can be determined experimentally using an OECD guideline or estimated based on QSAR relationships [128]. The cutoff for stability in water varies depending on the equilibrium times of the recommended methods, since shake-flask and column elution methods usually equilibrate in the time-range of day(s), whereas the slow-stir method equilibrate for week(s).

The methods suggested for the chemical groups vary in how well established they are in the literature. The two groups of chemicals covered within the 1995 OECD guidelines are the stable hydrophilic chemicals and stable solid hydrophobic chemicals (methods indicated in green). For the rest of the chemical groups, the methods are divided into methods, which are well described in literature and have been demonstrated to work for the designated chemical group (purple), and methods which are suggested based on literature descriptions using other chemicals or used for other purposes than solubility determinations (orange). It is therefore the expectation that the methods indicated by purple can be used without much alteration, whereas the methods indicated by orange require further developments and testing. The suggested methods for solubility determinations do not differ for the hydrophobic and very hydrophobic chemicals. However, the degree to which some of the methods have been demonstrated decreases for the very hydrophobic chemicals, and the degree of analytical challenges and third phase issues increase.

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Supplementary material: Examples of tracers used for spectroscopic methods.

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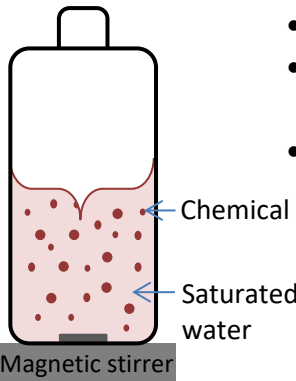
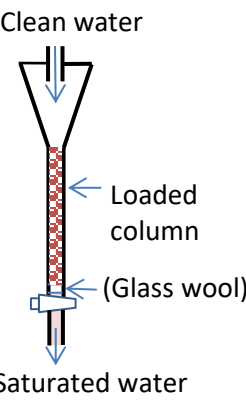
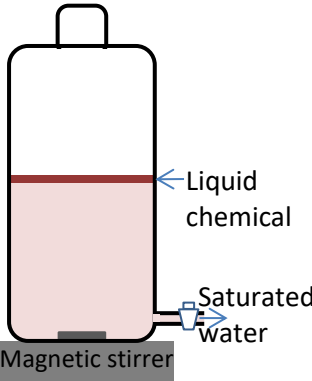
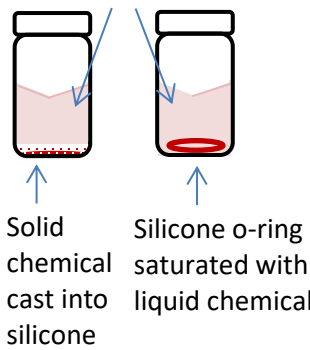
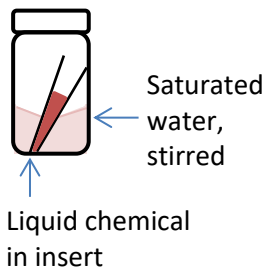
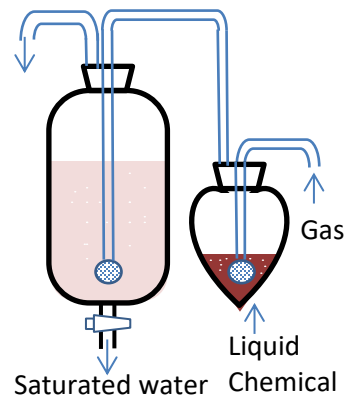
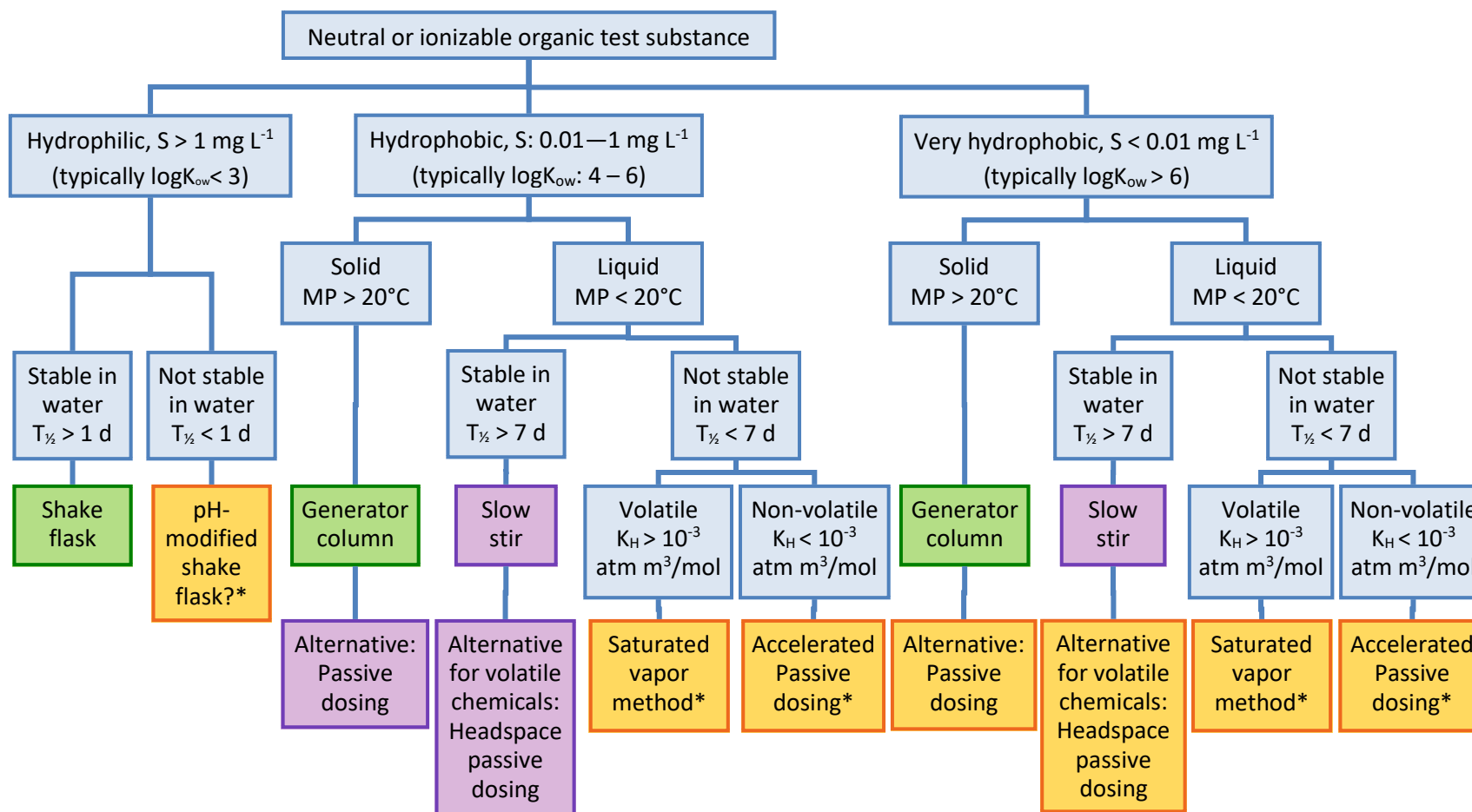
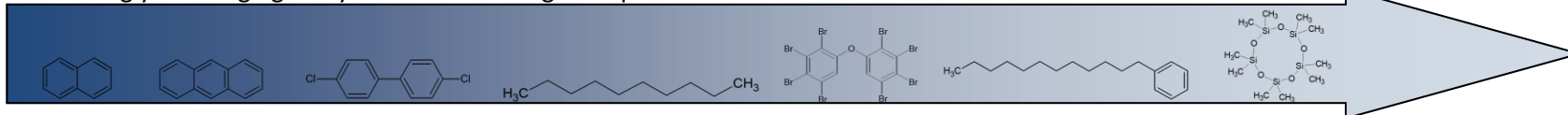
Current guideline saturation methods	
<p>Shake flask method</p>  <ul style="list-style-type: none"> • OECD 105, 1995 • Originated from: Solubility experiments • Applicable for hydrophilic chemicals <p>Labels: Chemical, Saturated water, Magnetic stirrer</p>	<p>Column elution/generator column methods</p>  <ul style="list-style-type: none"> • May et al. 1987 • OECD 105, 1995 • Originated from: Solubility experiments • Applicable for solid chemicals <p>Labels: Clean water, Loaded column, (Glass wool), Saturated water</p>
Other saturation methods	
<p>Slow stir method</p>  <ul style="list-style-type: none"> • Brooke et al., 1986 • Originated from: Determination of octanol-water partitioning, K_{ow} • Applicable for liquid chemicals <p>Labels: Liquid chemical, Saturated water, Magnetic stirrer</p>	<p>Passive dosing methods</p>  <ul style="list-style-type: none"> • Kwon and Kwon, 2012 • Smith et al., 2010a • Stibany et al., 2017 • Originated from: Toxicity testing • Applicable for hydrophobic chemicals <p>Labels: Saturated water, stirred, Solid chemical, Silicone o-ring saturated with liquid chemical</p>
<p>Headspace equilibration method</p>  <ul style="list-style-type: none"> • Trac et al., 2018 • Originated from: Toxicity testing • Applicable for liquid volatile chemicals <p>Labels: Saturated water, stirred, Liquid chemical in insert</p>	<p>Saturated vapor method (simplified)</p>  <ul style="list-style-type: none"> • Sanemassa et al., 1982 • Originated from: Solubility experiments • Applicable for liquid volatile chemicals <p>Labels: Saturated water, Gas, Liquid Chemical</p>

Figure 1: Conceptual drawing of selected methods for equilibrium based saturation of water. Temperature control measures, sample withdrawal and analysis are not included.



Increasingly challenging analytics and increasing third phase issues:



- 1
- 2 **Figure 2:** Decision tree for choice of solubility determination methods. Green indicates methods in the OECD guideline from 1995, purple
- 3 indicates methods demonstrated in current literature for the designated chemical group, orange indicates methods with a potential to
- 4 improve determinations for the chemical group, however not demonstrated for the designated chemical group in current literature.
- 5 Criteria for the groups are indicated in italics where *S* is the expected solubility, *logK_{ow}* is the octanol water partition ratio, *MP* is the

6 melting point, $T_{1/2}$ is the half-life in water, and K_H is the Henry's laws constant. Mixtures are not included, but methods can be chosen based
7 on their major constituents. *If the absence of stability stems from hydrolysis which is acid or base catalyzed, test should be performed at
8 pH at which test substance is most stable.