# 15

# Methods of Solvent Detection and Testing

## 15.1 STANDARD METHODS OF SOLVENT ANALYSIS

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This section includes information on solvent analysis based on methods included in national and international standards. ASTM standard methods are emphasized because they contain more methods on solvent testing than any other set of methods available. ISO standards are also covered in full detail because they are used in practical applications and are a basis for preparation of national standards. References to other national standards are also given including Australian (AS), Australian/New Zealand (AS/NZS), (British (BS), Canadian (CAN/CGSB), German (DIN), Japanese (JIS), and Finish (SFS). The same number is given in references to all national and international standards for the same solvent property to facilitate searching and referencing. Methods of analysis are grouped by subject in alphabetical order.

### 15.1.1 ALKALINITY AND ACIDITY

Amine acid acceptance by halogenated solvents is the degree to which an amine is capable of absorbing or neutralizing acid present from an external source or generated by the solvent.[1] This method is useful for comparing the effect of an amine with other acid-accepting compounds. The test is performed by the titration of an alkaline water extract from the solvent with 0.2 N hydrochloric acid to pH = 3.9 as detected by pH electrode.

The alkalinity of acetone is determined by a titration with 0.05 N $H_2SO_4$ in the presence of methyl red indicator.[2] The test method provides a measure of acetone alkalinity calculated as ammonia.

The acidity of halogenated solvents can be determined by titration with 0.01 N sodium hydroxide in the presence of a 0.1% solution of bromophenyl blue indicator.[3] Similar to the determination of alkalinity,[1] acidity is determined in water extract. The determination can also be done directly in solvent but the solution of sodium hydroxide should be prepared in methanol. A third option is to determine acidity by a pH-metric titration of a water extract. Prior to determination, the pH of solvent is measured. If the pH is above 7, then an alkalinity measurement is done by the above method.[1] If the pH is below 7, acidity is determined by this method.[3] The method is used to establish purchasing and manufacturing specifications and control the quality of solvents.

The acidity of benzene, toluene, xylenes, naphthas, and other aromatic hydrocarbons is determined by the titration of a water extract with 0.01 N sodium hydroxide in the presence of 0.5% phenolphthalein indicator solution.[4] The method is suitable for setting specifications, internal quality control, and development of solvents. The result indicates the potential corrosivity of solvent.

The acidity of solvents used in paint, varnish, and lacquer is determined by the titration of solvent diluted with water (for water soluble solvents) or isopropyl alcohol or ethanol (for water insoluble solvents) in proportion of 1:1. A water solution of 0.05 N sodium hydroxide in the presence of 0.5% phenolphthalein indicator dissolved in ethanol or isopropanol is used for titration.[5] The method is useful for determination of acidity below 0.05%. Acidity is a result of contamination or decomposition during storage, transportation or manufacture. The method is used to assess compliance with specification.

Solvents which are depleted of stabilizers (amine or alpha epoxide) may become acidic. The following method determines the combined effect of both alkaline (amine) and neutral (usually epoxy) stabilizers.[6] The determination is done in two steps. First solvent is mixed with hydrochlorination reagent (0.1 N HCl), then the excess is titrated with 0.1 N sodium hydroxide in the presence of 0.1% bromophenyl blue as an indicator.

The total acidity of trichlorotrifluoroethane and other halocarbons is determined by titration of a sample diluted with isopropanol with 0.01 N sodium hydroxide in isopropanol in the presence of a 0.05% isopropanol solution of phenolphthalein as an indicator.[7] The method is used for setting specifications and quality control.

### 15.1.2 AUTOIGNITION TEMPERATURE

The autoignition temperature can be determined by the hot and cold flame method.[8] Cool flames occur in vapor-rich mixtures of hydrocarbons and oxygenated hydrocarbons in air. The autoignition temperature is the spontaneous (self-ignition) temperature at which a substance will produce a hot flame without an external ignition source. Autoignition occurs when a hot flame inside a test flask suddenly appears accompanied by a sharp rise in temperature. With cold flame ignition the temperature rise is gradual.

The test equipment shown in a schematic drawing in the method[8] consists of a test flask, a furnace, a temperature controller, a syringe, a thermocouple and other auxiliary parts. The measurement is performed in a dark room for optimum visual detection of cool flames. The results are reported as ignition temperature, time lags (delay between sample insertion and material ignition), and reaction threshold temperature (the lowest flask temperature at which nonluminous pre-flame reactions (e.g., temperature rise) occur).

The results depend on the apparatus employed. The volume of vessel is especially critical. A larger flask will tend to produce lower temperature results. The method is not designed for materials which are solid at the measurement temperature or which undergo exothermic decomposition.

### 15.1.3 BIODEGRADATION POTENTIAL

The method covers a screening procedure which assesses the anaerobic biodegradation of organic materials.[9] The procedure converts organic substances into methane and carbon dioxide which are measured by a gas volumetric pipette – part of the standard apparatus. Other parts include, a biodegradation flask, a magnetic stirrer, a pressure transducer, a syringe, and a water seal. The apparatus may be interfaced with a gas chromatograph to determine quantities of the two gases.

The biodegradation process is conducted in a specially prepared medium inoculated with sludge inoculum. The process occurs under the flow of a mixture of 70% nitrogen and 30% carbon dioxide to provide anaerobic conditions.

The method was developed to screen organic substances for their potential to biodegrade. If a high degree of biodegradability is determined it provides a strong evidence that the test substance will be biodegradable in the anaerobic digestors of a waste treatment plant and in many natural environments. Other references[9] give methods of determining of biological and chemical oxygen demand.

### 15.1.4 BOILING POINT

The boiling point of solvent, its specific heat capacity, and its enthalpy of vaporization determine the energy required for solvent separation in a distillation column. They also determine numerous other properties of solvents (see Chapter 2).

Several methods of determination can be used, but two, distillation and gas chromatography are the most popular. Industrial aromatic hydrocarbons are determined by distillation. The temperature is recorded for the initial boiling point, for the sample which has been distilled at 5%, 10%, then at 10% increments up to 90%, then finally at 95%. The temperature should be recorded with precision of 0.1°C.[10] A general test method to determine the distillation range of volatile liquids[11] outlines a similar method of measurement. In addition to the measurements at the intervals given above, the temperature of the dry point (distillation temperature of residual quantities) is also recorded. Results must be reported as specified in the method.

A vacuum distillation procedure is used to determine the amount of solvents in solvent-based paints.[12] The paint sample is diluted with tricresyl phosphate, distilled for a while under normal pressure to evaporate the more volatile solvents, followed by vacuum distillation at 2 mm Hg.

Capillary gas chromatography is used to determine the boiling point of hydrocarbon solvents.[13] The initial boiling point is defined as the point at which the cumulative area of chromatogram equals 0.5% of its final total surface area. The final boiling point is at cumulative area of 95% of the total surface area of chromatogram. The method reports boiling point distribution in 1% intervals over the 1-99% range of the total cumulative surface area of chromatogram as well as the initial and final boiling points. A flame ionization detector is used in the determination and a standard solvent containing 16 known components is used for calibration.

### 15.1.5 BROMINE INDEX

Two methods are used to determine bromine index of aromatic hydrocarbons which contain trace amounts of olefins and are substantially free of materials lighter than isobutane and have distillation end-point lower than 288°C. The methods measure trace amounts of unsaturations in materials which have a bromine index below 500.

The bromine index can be measured by electrometric[14] and coulometric[15] titration. In the electrometric titration method, a sample is titrated with bromide-bromate solution (0.1 N solution of mixture of potassium bromide and potassium bromate) until the end-point increase in potential remains steady for 30 s.[14] In coulometric titration, a potassium bromide solution is used to titrate the solvent until the bromine concentration increases because it is no longer being consumed by the unsaturation of the solvent.[15]

Both methods can be used for setting specification, quality control, and testing of development solvents to find olefinic content. The methods do not differentiate between the types of unsaturations.

### 15.1.6 CALORIFIC VALUE

The heat of combustion of liquid hydrocarbon fuels can be determined with bomb calorimeter.[16] Two definitions are used in result reporting: gross heat of combustion (the quantity of energy released from fuel burned in constant volume with all products gaseous

except water which is in liquid state) and net heat of combustion (the same but water is also in a gaseous state). These determinations are useful in assessing the thermal efficiency of equipment used for generation of power or heat. The results are used to estimate the range of an aircraft between refueling stops which is a direct function of heat of combustion. The calorimeter bomb is standardized against benzoic acid standard. Net and gross heats of combustion are reported. A specific method is used for aviation fuels.[17] This method reports results in SI units and the measurements are made under constant pressure. The method is applicable for aviation gasolines or aircraft turbine and jet engine fuels. The method is used when heat of combustion data are not available. An empirical equation was developed which gives net heat of combustion based on the determined values of aniline point (ASTM D 611) and API gravity (ASTM D 287). If the fuel contains sulfur, a correction is applied for sulfur determined according to ASTM D129, D 1266, D 2622, or D 3120 (the method selected depends on the volatility of the sample).

Gross calorific value and ash content of waste materials can be determined by a calorimetric method.[19] After a calorimetric analysis, the bomb washing can be used to determine of mineral content by elemental analysis. The sample is burned under controlled conditions in oxygen. The calorimeter is standardized by burning known amount of benzoic acid. The formation of acids can additionally be determined by titration.

## 15.1.7 CLEANING SOLVENTS

Several standard procedures are available for evaluation of cleaning solvents. The stability of aircraft cleaning compounds is determined after 12 months storage at controlled conditions which may include moderate temperatures, cold storage, and hot storage.[20] Solvent vapor degreasing operations which use halogenated solvents follow standardized procedure.[21] The standard contains information on the location and design of a degreasing installation and operation during startup, degreasing, shutdown, and solvent reclamation. The purpose of the standard is to reduce the probability of accidents and exposure to personnel. A separate standard practice[22] gives reasons for and methods of preventing acid formation in degreasing solvents. The formation of acid is generally related to excessive heat, contaminations, the presence of chlorinated and sulfonated oils, admixture of acids, and solvent mixtures.

## 15.1.8 COLOR

Impurities in benzene, toluene, xylene, naphthas, and industrial aromatic hydrocarbons are determined by a simple colorimetric analysis of an acid wash.[23] A solvent is washed with sulfuric acid and the color of the acid layer is determined by a visual comparison with color standards prepared from solutions of cobalt chloride and ferric chloride.

Aromatic hydrocarbons which melt below 150°C can be subjected to color analysis in the liquid (molten) state using a visual comparison with platinum-cobalt standards.[24] Standards are prepared from a stock solution of $K_2PtCl_2$ to form a scale varying in color intensity. Similar procedure was developed to evaluate color of halogenated organic solvents and their admixtures.[25]

Objective color measurement is based on tristimulus colorimetry.[26] This instrumental method measures tristimulus values of light transmitted by a sample and compares the results to the values transmitted by distilled water. The results can be recalculated to the platinum-cobalt scale referred to in the previous standards.[24,25] The results can also be interpreted by normal methods of color measurement to yellowness index, color, color depth, etc.

### 15.1.9 CORROSION (EFFECT OF SOLVENTS)

The corrosiveness of perchloroethylene to copper is determined using Soxhlet apparatus.[27] Three pre-weighed strips of copper are used, one placed in the bottom flask, the second in the bottom of the Soxhlet attachment, and the third below the condenser. The specimens are exposed to refluxing solvent for 72 h after which the entire apparatus is flushed with distilled water to wash all acidic substances back to the flask. The water layer is titrated with 0.01 N NaOH to determine its acidity and the strips are weighed to determine weight loss. The results indicate quality of solvent. A different method is used to test copper corrosion by aromatic hydrocarbons.[28] Here, a copper strip is immersed in a flask containing solvent and the flask is placed in boiling water for 30 min. Next, the copper strip is compared with ASTM standard corroded copper strips.

If 1,1,1-trichloroethane is not properly stabilized it forms hydrochloric acid in the presence of aluminum. HCl corrodes aluminum. The presence of free water invalidates the result of this test.[29] An aluminum coupon is scratched beneath the surface of a solvent. The coupon is observed for 10 min and 1 h and the degree of corrosion is recorded in form of pass (no reaction) or fail (gas bubbles, color formation, or metal corrosion). The test is important to cleaning operations because aluminum should not be used for parts of machines (pumps, tanks, valves, spray equipment) in contact with corrosive solvent.

### 15.1.10 DENSITY

Density and specific gravity of solvents are discussed together. The difference in their definitions is that specific gravity is the density of material relative to the density of water whereas the density is the weight in vacuo of a unit volume. The density of liquids (including solvents) can be measured by a Bingham pycnometer.[30] The determination includes introduction of the liquid to the tared pycnometer, equilibration of temperature, then weighing. Other standardized method[31] determines the specific density of liquid industrial chemicals by two methods: hydrometer and pycnometer. The pycnometric method is essentially similar to the previously described. It differs in that the water and then solvent are weighed. Thus the density determination error may only be due to an imprecise weighing (the pycnometer calibrated volume does not enter calculations). In the hydrometer method, the calibrated hydrometer is immersed in controlled temperature liquid and direct readings are obtained.

The standard method for determining the specific gravity of halogenated organic solvents[32] involves the use of both a pycnometer and a hydrometer as described above but, in addition, an electronic densitometer is also used. Here, a liquid is placed in U-shaped tube and subjected to electronic excitations. The density changes the mass of tube and frequency of oscillations which is the basis for measurement and display of specific gravity readings.

Two standard tables[33,34] (American and metric system of units) are used to calculate weight and volume of benzene, toluene, xylenes mixture and isomers, styrene, cumene, and ethylbenzene as well as aromatic hydrocarbons and cyclohexane. Tables provide volume corrections for these solvents in a temperature range from -5 to 109°F (-20.5 to 43°C).

### 15.1.11 DILUTION RATIO

The dilution ratio is the maximum number of units of diluent that can be added to unit volume of solvent before precipitation occurs. Cellulose nitrate dissolved in an oxygenated solvent (8 wt% resin) is the most classical method to determine dilution ratio used to evaluate toluene as a standard diluent and to compare different diluents and solvents with a standard solvent (n-butyl acetate). The standard dilution ratio of toluene by n-butyl acetate

solution is 2.73-2.83. Two end points are determined. The first occurs when a known amount of diluent forms precipitate after 2 min of vigorous swirling. The second is determined by re-dissolving the precipitate, adding a known volume of solvent (dependent on dilution ratio) and precipitating it again with diluent.[35]

Similar method can be used for any resin, solvent, or diluent.[36] A solution of resin is prepared by the method described in ASTM D 1725 or by dispersing in blender (results may differ). Precipitation with diluent is determined at 25°C. The diluent is added dropwise from burette or weight is controlled throughout the experiment. The resin dilutability is recorded. If more than 100 g of diluent is required for 10 g of solution then diluent is regarded as being infinitely soluble.

Heptane miscibility in lacquer solvents is determined by mixing equal amounts of the specimen (lacquer) and heptane.[37] If a clear solution results after mixing, it indicates good miscibility. If a turbid solution results, either the heptane is immiscible with the tested specimen or water is present in either component.

Water-insoluble admixtures in solvents may affect many uses of solvent. The specimen solvent (primarily acetone, isopropanol, methanol, and many other) is diluted to 10 volumes with water and the resulting turbidity or cloudiness is recorded.[38]

### 15.1.12 DISSOLVING AND EXTRACTION

A standard practice for preparing polymer solution contains information on solvents, their concentration, temperature, pressure, mixing time, and heating.[39] The annex contains information on the best solvents for 75 typical polymers with different degrees of substitution or modification. Frequently, temperature and concentration of solution is also given.

Solvent extraction is used on textile materials to determine naturally occurring oily and waxy materials that have not been completely removed from the fibers.[40] The percentage of extracted material is given in relationship to the dry mass of fiber. Solvents used for extraction including 1,1,2-trichloro-1,2,3-trifluoroethane and dichloromethane but these may be replaced by other solvents by mutual agreement. The Soxhlet extraction and gravimetric determination are used.

### 15.1.13 ELECTRIC PROPERTIES

Specific resistivity is numerically equivalent to the volume resistance between opposite faces of one centimeter cube. The specific resistivity of electronic grade solvents is measured by a low-voltage a-c bridge operating at 1000 Hz providing the specific resistance does not exceed $10^9$ Ω-cm.[41] If the specific resistance is higher than $10^9$ Ω-cm, d-c equipment is used. D-c equipment is capable of measuring up to at least $10^{12}$ Ω-cm.

The electrical conductivity of liquid hydrocarbons can be measured by a precision meter.[42] The method is used to evaluate aviation fuels and other low-conductivity hydrocarbons. The generation and dissipation of electrostatic charges in liquids during handling depends on ionic species which may be characterized by electrical conductivity at rest. The dissipation time of the charges is inversely proportional to the conductivity. The measurement is done by a conductivity meter.

Two standard methods[43,44] were designed to determine the electric breakdown voltage of insulating oils of petroleum origin. These are VDE electrodes method[43] and the method under impulse conditions.[44] The dielectric breakdown voltage measurement allows to estimate the ability of an insulating liquid to withstand electric stress without failure. In the presence of contaminations such as water, dirt, or cellulosic fibers low breakdown voltages are obtained. VDE stands for Verband Deutscher Electrotechniker, an organization which designed brass electrodes used for measurement. The equipment uses a transformer, voltage

control equipment, and a voltmeter. The impulse method[44] uses highly divergent field under impulse conditions. The breakdown voltage of fresh oil decreases as the concentration of aromatic hydrocarbons increases. The method can be used for quality control of fresh oil, and determining the effect of service aging, and effect of impurities. The material is placed in test cell containing electrodes which are supplied from an impulse generator controlled by voltage control equipment.

### 15.1.14 ENVIRONMENTAL STRESS CRAZING

Crazing is a group of surface fissures which appear as small cracks after the material has been exposed to solvent and stress. Crazes are usually oriented perpendicular to stress and their appearance depends on the index of refraction and on the angle of viewing. A suitable light source must be used. Transparent plastic materials can be directly tested for crazing.[45] Two variations of method are used to determine stress crazing of transparent plastics: determination of stress required to cause cracking or determination of craze development along the time of stress application. In the first method specimens are exposed to solvent by the direct contact of specimen surface with filter paper wetted with solvent. By selecting different values of stress (using each time new specimen) the range of two stress forces is searched for the largest stress under which specimen does not craze and the smallest stress under which it does craze. In the second variation of the method, sample is tested first without solvent to assure that it does not craze under the selected load. Solvent is then applied and specimen inspected in 15 min time intervals taking note of location of craze front as crazing progresses. If the sample does not craze higher load is selected and *vice versa*. Similar to the first variation the range is determined within which specimen crazes and does not craze.

The crazing effect can be indirectly determined by testing chip impact resistance of specimens which crack either because of weathering or environmental stress cracking.[46]

### 15.1.15 EVAPORATION RATE

The evaporation rate of a solvent is determined to obtain relative value to some standard, selected solvent. The solvent selection depends on reasons for solvent use and the type of solvent and it is usually agreed upon between interested parties. In Europe, diethyl ether is the most frequently used reference solvent and in the US butyl acetate. The evaporation rate of other solvents is determined under identical conditions and the resultant values are used to rank solvents. The most obvious requirement is that the determination is done without excessive drafts and air currents. The evaporation rate is the ratio of the time required to evaporate a test solvent to the time required to evaporate the reference solvent under identical conditions. The results can be expressed either as the percentage evaporated within certain time frame, the time to evaporate a specified amount, or a relative rate. Relative rate is the most common.

For halogenated solvents used in cleaning applications, the relative evaporation rate is compared either to xylene or perchloroethylene.[47] The determination is done on a test panel using 10 ml of solvent. The relative evaporation rate is calculated.

### 15.1.16 FLAMMABILITY LIMITS

These tests cover the methods of determination of the minimum temperature at which vapors in equilibrium with liquid solvent are sufficiently concentrated to form flammable mixtures with air at atmospheric pressure and concentration limits of chemicals. Flammable (explosive) limits are the percent levels, volume by volume, of a flammable vapor or gas mixed in air between which the propagation of a flame or an explosion will occur upon the

presence of ignition. The leanest mixture at which this will occur is called the lower flammable limit. The richest mixture at which it will occur is the upper flammable limit. The percent of vapor mixture between the lower and upper limits is known as the flammable range.

The temperature limits of flammability can be determined in an air/vapor mixture above a flammable liquid in a closed vessel.[48] The temperature in vessel is varied until a minimum temperature is reached at which the flame will propagate away from the ignition source. A glass vessel is equipped with an ignition device, a magnetic stirrer, a clamping devices, and a safety glass window. The initial temperature of determination is estimated from closed-cup flash point measurement. If the flash point is below 38°C, the initial temperature should be 8°C below flash point temperature. If the flash point is between 38 and 96°C, the starting temperature should be at least 14°C below the flash point. If the flash point temperature is above 96°C then the initial temperature should be 22 to 44°C below the flash point temperature. Selecting higher initial temperature may result in explosion. The lower temperature limit of flammability is obtained from the test which can be used to determine guidelines for the safe handling of solvents in closed process and storage vessels.

The concentration limits of flammability are determined using another method.[49] The method is limited to atmospheric pressure and temperature of 150°C. Equipment is similar to that used in the previous method. A uniform mixture of vapor and air is ignited and flame propagation from ignition source is noted. The concentration of flammable components is varied until a composition is found which is capable to propagate flame.

### 15.1.17 FLASH POINT

A variety of apparatus such as the small scale closed tester,[50] the tag open cup,[51] the tag closed tester,[52] the Cleveland open cup,[53] the Pensky-Martens closed cup,[54] and the equilibrium method[55] are used to determine the flash point. The selection of method is based suggestions included in separate standard.[56]

The small scale closed tester[50] is a metal cup with a thermometer fitted below the bottom of the internal chamber with a hinge mounted cover having filling orifice. The sample is introduced to the cup and the cup is maintained at a constant temperature by means of temperature controller. After a specific time, a test flame is applied for 2.5 s and an observation is made whether or not flash has occurred. If flash did not occur the cup is cleaned, a new sample is introduced and the temperature is increased by 5°C. The measurements are repeated until the flash point is determined with accuracy of 1°C.

The tag open cup[51] is a larger unit equipped with water-glycol bath for temperature control or a solid carbon dioxide-acetone bath for lower flash points. A much larger sample is used with this equipment and the temperature is gradually increased at a rate of 1°C/min. A taper flame is passed for 1 s in 1 min intervals until the flash point is detected. The tag closed tester[52] can use either a manual or an automated procedure. A sample volume of 50 ml is used. Either a gas flame or an electric ignitor is used. In the automated mode, the equipment is programmed to perform the standard procedure.

The Cleveland open cup[53] is placed on a heated plate which increases temperature at a rate of 5-6°C/min. This method can also be automated. The method is designed for testing petroleum products which are viscous and have flash point above 79°C. The Pensky-Martens[54] closed cup tester was also designed for petroleum products but for those with flash points from 40 to 360°C. This apparatus has its own heating source, stirrer, and cover by which it differs from Cleveland cup. It can be either manual or automated. The equilibrium method[55] uses either a modified tag close cup or the Pensky-Martens apparatus. The modification intended to keep the vapor/air temperature in equilibrium with the liquid temperature. The method is limited to the temperature range from 0 to 110°C.

Depending on the viscosity of liquid and its expected flash point range, one of the above methods is chosen as described in detail elsewhere.[56] It should be additionally noted that if the flash point method uses continuous heating, it is not suitable for testing mixtures of flammable substances because their vapor concentrations are not representative of equilibrium conditions. One of the weaknesses of flash point analysis is that the flame is well above the liquid surface therefore full vapor concentration is not attained. Many cases exist where a flash point cannot be detected but the material does form flammable mixtures. Before a method is chosen and a data interpretation made full information on the test procedure should be studied in detail and the proper authorities should be consulted to define safe practices for a particular material.

### 15.1.18 FREEZING POINT

Freezing point apparatus consists of freezing tube, Dewar flasks to act as cooling and warming baths, stirring mechanisms, absorption tubes, clamps and other auxiliary parts.[57] Freezing point can be obtained precisely from interpretation of time-temperature freezing and melting curves. The determination is made by measuring the electrical resistance of liquid which decreases on cooling and becomes constant when it freezes. This method in conjunction with the testing details described in a separate standard[58] can be used to determine the purity of many hydrocarbon solvents. The data given in the last standard[58] allow for a precise determination of the purity of solvent in percent of pure compound. A simple method was designed to determine solidification point of benzene based on visual observation of formation of solid phase.[59]

### 15.1.19 FREE HALOGENS IN HALOGENATED SOLVENTS

This simple qualitative test involves the extraction by water of free halogens, followed by the reaction of the halogens with potassium iodide in the presence of a starch indicator.[60] The solution color changes to blue in the presence of free halogens.

### 15.1.20 GAS CHROMATOGRAPHY

Gas chromatography provides many tools for the analysis of solvents. In section 15.1.4, a method was discussed which determines the distribution of boiling points of hydrocarbon mixtures. Many uses of gas chromatography for the determination of purity of different solvents are discussed in Section 15.1.25. In this section, some examples of of gas chromatography are included to show its usefulness in the qualitative determination of solvents mixtures[61] the analysis of solvent impurities,[62-64] the determination of solvents in a product by direct injection,[65] and the generation of data to evaluate waste materials to determine their hazardous content.[66]

The relative distribution of aromatic hydrocarbons in xylene products can be quantitatively determined by gas chromatography.[61] A flame ionization or thermal conductivity detector is used with a capillary or packed column containing crosslinked polyethylene glycol as the stationary phase. The peak area of each component is measured and the weight percentage concentration is calculated by dividing the peak area of the component by the sum of the areas of all peaks.

Ortho-xylene concentration and the concentrations of its admixtures and impurities are measured using a flame ionization detector and a polar fused-silica capillary column.[62] An internal standard (iso-octane) is used to increase precision and a standard mixture is used for calibration. A similar method is used with p-xylene but either n-undecane or n-octane are used as the internal standards.[63] In both methods, peak areas are interpreted relative to the peak area of internal standard. The main impurities in benzene are non-aromatics with less than 10 carbons, toluene, 1,4-dioxane and aromatics containing 8 carbon atoms. The

method of determination of benzene impurities[64] is similar to two methods described above.[62,63] Normal-nonane is used as an internal standard. In all three methods the internal standards must be at least 99% pure component.

A gas chromatograph equipped with a thermal conductivity or a flame ionization detector and capillary or packed columns is used for direct determination of solvents in paints.[65] Columns are usually packed with either polyethylene glycol (molecular weight 20,000) or a diisodecyl phthalate as liquid phase on diatomaceous earth (60-80 mesh) used as a solid support. Low viscosity paints are drawn into a syringe and injected through the injection port. High viscosity paints are diluted with solvent that does not interfere with the analysis, usually ethyl ether or dichloromethane. A standardized gas chromatographic technique[66] is capable of determining the 67 solvents most frequently found in hazardous wastes. Several detectors are suggested for analysis such as flame ionization, electron capture, thermal conductivity, photoionization, or mass selective. Each waste mixture may contain a large number of solvents. Their detection is facilitated by the use of gas chromatograph interfaced with mass spectrometer. The method is designed to facilitate site assessment, recycling operations, plant control, and pollution programs.

## 15.1.21 LABELING

Warnings, first aid measures and operating instructions are standardized for vapor degreasers.[67] Placards containing this information should be placed close to the degreaser in an area accessible to employees. The placards should contain the information required by applicable federal and local laws and regulations. The placard should include name of the solvent used, and warnings indicating that the vapor is harmful, that breathing the vapor should be avoided, that the machine should only be used with proper ventilation, that swallowing and contact with the skin should be avoided, that cutting or welding should not be performed close to the machine, and that the tank should not be entered unless a proper procedure is followed. In addition, start-up and operation procedures should be available.

Minimum labeling requirements for several halogenated solvents should also follow a standard practice.[68] The label should state the company name, its logo and address, emergency telephone numbers, lot number, the net weight, solvent name, its CAS number, OSHA PEL and ACGIH TLV values, and quantity. In addition, health and safety information, precautions, first aid, and handling and storage information should be provided.

## 15.1.22 ODOR

Strips of rapid qualitative paper are dipped in a standard liquid and in the liquid under the test. Their odor is compared to establish if the odor of sample is more or less acceptable than the standard to the purchaser and the manufacturer.[69] In a similar method, papers dipped in a standard and specimens are allowed to dry at room temperature and tested for residual odor at specified time intervals.

Odor testing may be performed by a selected group of panelist to either determine the effect of various additives on the odor or taste of a medium or to determine the odor or taste sensitivity of a particular group of people.[70] For this purpose, a series of samples is prepared in concentration scale which increases in geometric increments. At each concentration step two samples containing the medium alone are given to a panelist. The panelist should determine which sample is different from the other two samples. The panelist should begin with the lowest concentration selected to be two or three concentration steps below estimated threshold. The method description contains information on sample selection and preparation, result, and precision determination.

A method of evaluation of denatured and undenatured alcohols to assess their acceptability is used.[71] This method is developed specifically to compare methanol, ethanol,

isopropanol, and n-propanol. The group of panelist is asked to compare the characteristic and residual odors of evaporation, its intensity by dilution, and its concentration. A similar method was developed for a series of glycols.[72] Here, odor character and intensity are evaluated.

The residual odor of a drycleaning grade of perchloroethylene is determined by comparing treated and untreated samples of bleached cotton fabric. The treated fabric is soaked for 5 min in perchloroethylene and dried for 4 hours at room temperature.[73] Good quality perchloroethylene should leave no odor.

### 15.1.23 PAINTS – STANDARDS RELATED TO SOLVENTS

The paint industry, a major user of solvents, has developed numerous standards. Some are included in Sections 15.1.25, 15.1.31, and 15.1.35. The paint industry also uses many general standards and some specific standards, which have not been included in any other section of this chapter. Details of these are given below.

Sampling and testing requirements for solvents used in the paint industry are summarized in a special standard.[74] This comprehensive list of standards used by paint industry also includes a brief discussion of each method of testing, including sampling, specific gravity, color, distillation range, nonvolatile matter, odor, water, acidity, alkalinity, ester value, copper corrosion test, sulfur, permanganate time test for acetone and methanol, flash point, purity of ketones, solvent power evaluation, water miscibility, analysis of methanol, analysis of ethylene and propylene glycols, acid wash color of aromatic hydrocarbons, paraffins and other nonaromatic hydrocarbons in aromatics, and aromatics in mineral spirits.

The nonvolatile matter in paints is determined by a gravimetric method after drying a 100 ml sample in oven at 105°C.[75] The transfer efficiency of paints is a volume or weight ratio of paint solids deposited to the volume/weight of the paint solids sprayed, expressed in percent. This method[76] can be used to optimize the paint application process. The measurement is done by weighing or measuring the volume of paint used on a certain sprayed surface area and comparing this value with known or predetermined by the above method weight of solids in the paint used for spraying.

In order to determine an ester value for solvents or thinners, the specimen is reacted with aqueous potassium hydroxide, using isopropanol as the mutual solvent.[77] The hydrolysis is conducted at 98°C and the excess potassium hydroxide is determined by titration. From the amount of potassium hydroxide consumed, the ester value is calculated.

### 15.1.24 pH

A method developed for halogenated solvents is applicable for determining the pH of water extracts of solvents.[78] The solvent sample is shaken with distilled or deionized water and the pH is determined either by comparing color upon the addition of Gramercy universal indicator or by using a glass electrode pH meter.

### 15.1.25 PURITY

Several techniques are used to determine purity of solvents. Gas chromatography is the most common and this and other methods are discussed first followed by other analytical methods which include instrumental and simple methods. The aim of these tests is to determine the concentration of the main component but more frequently qualitative and quantitative determination of admixtures. Some methods have already been discussed in Sections 15.1.1, 15.1.5, 15.1.8, 15.1.9, 15.1.18, 15.1.20 and 15.1.23.

Alcohol content and purity of an acetate ester is determined by gas chromatography.[79] The method was applied to ethyl, n-propyl, isopropyl, n-butyl, isobutyl, and 2-ethylhexyl acetates. Water and acetic acid cannot be measured by this method and other methods are

used. A thermal conductivity or flame ionization detector is used. A stainless steel column with 80-100 mesh Chromosorb G-HP is used with 9.05% Dow Corning QF-1 and 0.45% Igepal CO-990. The concentration of the main component and the amount of free alcohol are measured by the method.

Traces of benzene in hydrocarbon solvents are measured by capillary gas chromatography.[80] Because of the hazardous nature of benzene, the method was introduced to ensure compliance with the stringent regulations. A flame ionization detector is used with 0.53 mm fused silica capillary columns with bound methyl silicone or polyethylene glycol. Similar method is used to determine benzene content in cyclic products (cyclohexane, toluene, cumene, styrene, etc.).[81] This method does not specify any particular column but the column used should be able to resolve benzene from other components. The method can determine benzene in concentrations of 5 to 300 mg/kg. Traces of thiophene in refined benzene are determined by a flame photometric detector.[82] Several column types given in standard are found satisfactory to overcome potential problem of quenching effects of hydrocarbons on the light emissions from thiophene. High purity benzene for cyclohexane feedstock is tested for several known impurities by capillary gas chromatography.[83] The gas chromatograph is equipped with a flame ionization detector and a splitter injector suitable for fused silica capillary column internally coated with crosslinked methyl silicone. The concentration of benzene and the concentrations of impurities can be adequately determined.

The purity of halogenated solvents is determined using a thermal conductivity or flame ionization detector, a column made from 3.2 mm stainless tubing packed with 30 wt% silicone fluid on 80-100 mesh diatomaceous earth or using capillary column.[84] Admixtures in 1,1,1-trichloroethane are determined using a thermal conductivity or hydrogen flame detector. Column from copper or stainless steel is packed with Chromosorb W HP with 20% polydimethylsiloxane.[85]

Various impurities, such as hydrocarbons, acetone, alcohols and other can be determined using a thermal conductivity or a flame detector.[86] Several columns are specified in the standard mostly using polyethylene glycol on diatomaceous earth. For determination of purity of methyl isobutyl ketone different method is used.[87] In both cases the amounts of determined impurities are subtracted from total mass to give purity of specimen.

The determination of aromatics in mineral spirits is another method that has been developed to ensure compliance with regulations restricting aromatic content.[88] Three methods are given, each capable to determine ethylbenzene and total aromatic content. The methods differ in column type and packing.

A spectrophotometric method[89] for determining thiophene in benzene is available as alternative to gas chromatography.[82] The spectrometer used is capable of detecting absorbance in the range from 400 to 700 nm with a repeatability of 0.005 absorbance units. Thiophene is reacted with isatin to form a colored compound. The quantitative determination is based on reading concentrations from master curve.

The presence of reducing substances in pyridine can be detected by a simple visual observation.[90] The sample of pyridine is mixed with 0.32% potassium permanganate solution and color is observed after 30 min. If color of the potassium permanganate is retained, the sample is free of reducing agents.

Oxidative microcoulometry is used to determine trace quantities of sulphur in aromatic hydrocarbons.[91] An oxidative pyrolysis converts sulfur to sulfur dioxide which is titrated in titration cell with the triiodide ion present in the electrolyte.

The total chloride (organic and inorganic) in aromatic hydrocarbons and cyclohexane can be determined by titration.[92] Bromides and iodides present are recorded as chlorides. The sample is mixed with toluene in a proportion which depends on the expected concentration of chloride. The reagent sodium biphenyl is added to convert organic halogens into in-

organic halides. After decomposing the excess reagent with water, the separated aqueous phase is titrated in presence of acetone with a silver nitrate solution. Organic chlorides present in aromatic hydrocarbons can also be determined by microcoulometry.[93] The presence of chlorine compounds may adversely affect equipment and/or reaction therefore their concentration is frequently controlled. A liquid specimen is injected into a combustion tube maintained at 900°C, converted to hydrogen halides, and carried by a carrier gas (50% oxygen, 50% argon) to a titration cell where it reacts with silver ions in the electrolyte.

The determination of peroxides has two goals: one is to monitor peroxide concentration used as initiator and catalysts and the other is to detect formation of hazardous peroxides formed as autoxidation products in ethers, acetals, dienes, and alkylaromatic hydrocarbons. A sample is dissolved in a mixture of acetic acid and chloroform. The solution is deaerated and potassium iodide reagent is added and let to react for 1 h in darkness.[94] The iodine formed in reaction is measured by absorbance at 470 nm and result calculated to active oxygen in the sample. The method can determine hydroperoxides, peroxides, peresters, and ketone peroxides. Oxidizing and reducing agents interfere with the determination.

Mercaptans in motor fuels, kerosene and other petroleum products can be detected by shaking the liquid sample with sodium plumbite solution, adding powdered sulfur and shaking again.[95] If a mercaptan or hydrogen sulfide is present, discoloration of the floating sulfur or liquid phase occurs.

The nonvolatile content of a halogenated solvent is determined by drying the sample in a platinum evaporating dish at 105°C.[96] Depending on boiling point and the concentration of nonvolatile matter three alternate procedures are proposed.

The Karl Fisher method is recommended for general use in solvents to determine the water content.[97] It is not suitable if mercaptans, peroxides, or appreciable quantities of aldehydes and amines are present. Water in halogenated solvents may cause corrosion, spotting, reduce shelf-life of aerosols, or inhibit chemical reactions, thus special method, also based on the Karl Fischer titration, was developed for halogenated solvents.[98]

The titration of ionizable chlorides with mercuric acetate solution in the presence of s-diphenylcarbazone as an indicator is used to determine chloride in trichlorotrifluoroethane.[99] A visual appearance test to detect admixtures in halogenated solvents is based on the observation of suspended particles, sediment, turbidity and free floating water.[100]

The aromatic content of hydrocarbon mixture is estimated from the determination of aniline point.[101] Aromatic hydrocarbons have the lowest and paraffins the highest aniline points. Cycloparaffins and olefins are between the two. Aniline point increases as the molecular weight increases. A mixture of specific aniline and solvent is heated at a controlled rate until it forms one phase. The mixture is then cooled and the temperature at which the miscible liquid separates into two phases is determined. Four methods are discussed in the standard[101] suitable for transparent, non-transparent, easily vaporizing, and measured in small quantities.

The presence of oxidizable materials in acetone and methanol that are associated with contaminations during manufacture and distribution can be evaluated by permanganate time.[102] Oxidizable contaminants may adversely affect catalysts or ligand complexes which are sensitive to oxidation. Oxidizable substances reduce potassium permanganate to manganese oxide which is yellow. The method is designed to measure the time required to change color to the color of a standard.

Small admixtures of acetone in methanol (more than 0.003 wt%) can be detected after a reaction with Nessler's reagent.[103] The reacted sample is compared with a standard, which contains 0.003 wt% acetone and the difference in turbidity is reported.

## 15.1.26 REFRACTIVE INDEX

Refractive index is measured by a standard method.[104] It covers transparent and light colored liquids having a refractive index in the range from 1.33 to 1.50. The refractive index is the ratio of light velocity of a specified wavelength in air to its velocity in the substance under evaluation. The refractive dispersion is the difference between refractive indices for light of two different wavelengths. This value is usually multiplied by 10,000. The method uses a Bausch & Lomb refractometer equipped with a thermostat and with a circulating bath to control the sample temperature with a precision of 0.2°C. Several light sources can be used, including the sodium arc lamp, mercury light lamp, and hydrogen or helium discharge lamp. Light sources are equipped with filters which transmit a specific spectral line. Standardization of equipment is done using a solid reference standard or using liquids standards such as 2,2,4-trimethylpentane (1.39), methylcyclohexane (1.42), or toluene (1.49).

## 15.1.27 RESIDUAL SOLVENTS

Residual solvents may cause odor, off-taste, blocking, and an increased degradation rate in outdoor exposures. A single standard test method has been developed to determine residual solvent levels and it is primarily used for the evaluation of flexible barrier materials.[105] The method is based on gas chromatography. The specimen of the barrier material is enclosed in a container and heated to vaporize the retained solvents into the head space. The vapor from the head space is taken by a gas syringe and injected into a gas chromatograph. The recovery of solvents is compared by the means of response factor which is a peak intensity of the detector in response to a given volume of injected sample. Response factors of different solutions vaporized in the test containers are compared. Round robin tests have demonstrated that this method has a coefficient of variation between laboratories of ±15%. The method does not specify the detector or the columns. Before a flexible barrier material is analyzed, the optimum heating time to recover volatilized solvents is determined.

## 15.1.28 SOLUBILITY

Solvent power of a hydrocarbon solvents is determined by kauri-butanol value.[106] The method applies to solvents having a boiling point above 40°C and a dry point below 300°C. The method is most frequently used to evaluate solvents for applications in paints and lacquers. The kauri-butanol value is the volume of solvent required to produce a defined degree of turbidity when added to 20 g of a standard solution of kauri resin in n-butanol (400 g of resin in 2000 g of solvent). High kauri value indicates a relatively strong dissolving power. The method is standardized using 105±5 ml of toluene.

The solubility of common gases in hydrocarbon liquids is determined to meet requirements of aerospace industry.[107] This test method is based on the Clausius-Clapeyron equation, Henry's law, and the perfect gas law. The results are important in the lubrication of gas compressors where dissolved gas may cause erosion due to cavitation. In fuels, dissolved gases may cause interruption of fuel supply and foaming in tank. The liquid density is determined experimentally. Using this density, the Ostwald coefficient is taken from a chart and used for the calculation of the Bunsen coefficient (solubility of gas). The solubility of the gas or mixture of gases and Henry's law constant are also calculated.

## 15.1.29 SOLVENT PARTITIONING IN SOILS

A procedure is available to determine partitioning organic chemicals between water and soil or sediment.[108] By measuring sorption coefficients for specific solids, a single value is obtained which can be used to predict partitioning under a variety of conditions. The

underlining principle of the method is that organic chemicals bind with the surfaces of solids through chemical and physical interactions. However, the sorption coefficient is based on organic carbon content which does not apply to all solvents or all soils.

The sorption coefficient of a particular solvent is measured by equilibrating its aqueous solution, containing a realistic concentration similar to that found in the environment, with a known quantity of soil or sediment. After equilibrium is reached, the concentration of solvent in the water and the soil is measured by a suitable analytical technique. The sorption constants for all solids tested are averaged and reported as a single value. The standard does not define the actual method of determining the concentrations but strategy that should be followed. The data are useful in predicting the migration of chemicals in soil, in estimating their volatility from water and soil, determining their concentration in water, and their propensity to leach through the soil profile.

### 15.1.30 SOLVENT EXTRACTION

Often materials must be extracted from a compounded product to perform testing. Extracted material must then be recovered from solution without degradation to be subjected to testing. The method discussed here was developed as means of recovering asphalt from pavement samples.[109] The solution of extracted asphalt in solvent is distilled by rotating the distillation flask of a rotary evaporator in a heated oil bath. The distillation is carried out under partial pressure in the presence of nitrogen to prevent degradation. The asphalt recovered by this method can be tested in the same manner as were the original asphalt samples.

### 15.1.31 SPECIFICATIONS

Standard specifications are designed to set criteria for commercial solvents which can be used to determine the compliance of a solvent sample. Because applications of solvents differ very widely, the selected criteria are also different for different groups of solvents. Table 15.1.1 is a compiled list of parameters all of which can be found in solvent specifications. The most common parameters used to characterize solvent include acidity, appearance, color, concentration of main component, distillation range, dry point, initial boiling point, and specific gravity. The methods of determining these parameters are found in this chapter.

The list of references includes information on specifications for various solvents. Solvents in this list are arranged into groups: alcohols,[110-120] aromatic hydrocarbons,[121-128] other hydrocarbons,[129-132] ketones,[133-138] esters,[139-145] glycol,[146] and chlorine-containing solvents.[147-152]

### 15.1.32 SUSTAINED BURNING

The sustained burning test was originally developed for British Standard BS-3900 and adapted by ASTM.[153] The purpose of the test is to determine the sustained burning characteristics of solvents by direct experiment rather than by deducing characteristics from flash point data. Mixtures of some flammable liquids (e.g., alcohol and water) are classified as flammable based on the closed-cup flash point method. Some mixtures may be classed as flammable even though they do not sustain burning. The test is performed in a block of aluminum with a concave depression called a well. The liquid under test is heated to a temperature of 49°C and a flame is passed over the well and held in position for 15 s. The specimen is observed to determine if it can sustain burning.

**Table 15.1.1. Typical chemical and physical properties of solvents included in their specifications**

| | |
|---|---|
| Acid acceptance | Distillation range |
| Acidity | Doctor test |
| Acid wash color | Dry point |
| Alkalinity | Flash point |
| Aluminum scratch | Free halogens |
| Aniline point | Initial boiling point |
| Appearance | Iron concentration |
| Bromine index | Kauri-butanol value |
| Color | Non-volatile matter |
| Concentration of admixtures | Permanganate time |
| Concentration of isomers | Residual odor |
| Concentration of main component (or purity) | Specific gravity |
| Concentration of sulfur | Water concentration |
| Copper corrosion | Water miscibility |

## 15.1.33 VAPOR PRESSURE

The vapor pressure is the pressure of the vapor of a substance in equilibrium with the pure liquid at a given temperature. Two procedures are used: an isoteniscope procedure (standard) for measuring vapor pressures from $1x10^{-1}$ to 100 kPa and a gas-saturation pressure for measuring vapor pressures from $1x10^{-11}$ to 1 kPa.[154-155] In the isoteniscope method, a sample is deaerated by heating under reduced pressure. The vapor pressure is then determined by balancing the pressure of the vapor against a known pressure of an inert gas. The vapor pressure is determined at minimum three different temperatures.

In the gas-saturation method, an inert gas is passed through a sufficient amount of compound to maintain saturation. The vapor is then removed from the gas by a sorbent or a cold trap and quantitatively determined by gas chromatography or other suitable technique.

## 15.1.34 VISCOSITY

The viscosity of solvents can be determined by one of three methods: glass viscometer,[156] Saybolt viscometer,[157] and bubble time method.[158] Glass viscometry is applicable to Newtonian, transparent liquids which because of volatility cannot be measured in conventional capillary viscometers. The viscometer uses a purge gas which helps to transfer the measuring liquid from a lower reservoir to the sample bulb. The time of flow is measured for a fixed volume of liquid at a temperature controlled with a precision of 0.01°C. A set of liquids is available as viscosity standards in order to select the standard having closest viscosity to the measured sample.

The Saybolt viscometer was developed for petroleum products. A sample of 60 ml flows through a calibrated orifice and the time of flow is measured at a controlled tempera-

ture. Measurements are made at a temperature selected from the range 21-99°C. The instrument is standardized by measuring the flow of a standard oil at both 37.8 and 50°C.

In the bubble method, a standard viscosity tube is filled with a specimen liquid, the temperature of liquid is equilibrated to 25°C in a bath, the level of the meniscus is adjusted to 100 mm line, cork is inserted to end on 108 mm line, sample is hold in thermostating bath for another 20 min. Then the tube is inverted and the time for the bubble to flow from a mark at 27 mm to 100 mm is measured.

### 15.1.35 VOLATILE ORGANIC COMPOUND CONTENT, VOC

Several terms are used in the paint industry to provide data on the VOC content of paints.[159] The percent of solids in paints is calculated either per unit weight or per unit volume. The weight solids content is the weight of non-volatile materials divided by the total weight of the coating. In practice, it is calculated by subtracting the total weight of volatile solvents from the total weight of the coating and dividing the result by the total weight of coating. The final result is multiplied by 100 to express it in percent. Percent of solids by weight is calculated in similar manner.

A solvent, as defined by paint standards, is a volatile liquid that is incorporated primarily for vehicle solvency and control of the application characteristics. The solvent content is the calculated weight of solvents in a specific volume of paint. This definition is not equivalent to the definition of VOC compound because it does include compounds which are excluded by EPA (see more on this subject in Section 18.1).

Volatile organic compound by EPA definition means any compound of carbon, excluding carbon monoxide, carbon dioxide, carbonic acid, metallic carbides or carbonates, and ammonium carbonate, which participates in atmospheric photochemical reactions. This includes any such organic compound other than the following, which have been determined to have negligible photochemical reactivity: methane; ethane; methylene chloride; 1,1,1-trichloroethane; 1,1,2-trichloro-1,2,2-trifluoroethane; trichlorofluoromethane; dichlorodifluoromethane; chlorodifluoromethane; trifluoromethane; dichlorotetrafluoroethane; chloropentafluoroethane; dichlorotrifluoroethane; tetrafluoroethane; dichlorofluoroethane; chlorodifluoroethane; 2-chloro-1,1,1,2-tetrafluoroethane; pentafluoroethane; 1,1,2,2-tetrafluoroethane; 1,1,1-trifluoroethane; 1,1-difluoroethane; perchloroethylene; acetone; parachlorobenzotrifluoride; cyclic, branched, or linear completely methylated siloxanes; and perfluorocarbon compounds which fall into these classes: cyclic, branched, or linear, completely fluorinated alkanes; cyclic, branched, or linear, completely fluorinated ethers with no unsaturations; cyclic, branched, or linear, completely fluorinated tertiary amines with no unsaturations; and sulfur containing perfluorocarbons with no unsaturations and with sulfur bonds only to carbon and fluorine. For purposes of determining compliance with emissions limits, VOC is measured by the test methods in the approved State implementation plan (SIP) or 40 CFR Part 60, Appendix A, as published in (7/1/91) edition, as applicable.

Based on the above definition of VOC, the VOC content is calculated from a formula by excluding from the total solvent content of the paint, the content of water and solvents excluded by the above regulation. The following formula can be used for VOC calculation:

$$VOC = \frac{W_{total\ solvents} - W_w - W_{excluded\ solvents}}{V_{paint} - V_w - V_{excluded\ solvents}} \qquad [15.1.1]$$

where:

| | |
|---|---|
| $W_{total\ solvents}$ | total weight of solvents |
| $W_w$ | weight of water present in formulation |

| | |
|---|---|
| $W_{excluded\ solvents}$ | total weight of solvents excluded by EPA regulation |
| $V_{paint}$ | total volume of paint |
| $V_w$ | volume of water present in formulation |
| $V_{excluded\ solvents}$ | total volume of solvents excluded by EPA regulation |

The above definition may vary in various countries thus the proper definition should be obtained from the appropriate authorities. Examples of other definitions are included in Chapter 18.1.

The weight percent of the volatile content in waterborne aerosol paints is determined by releasing the propellant from can, testing the remaining paint for water content using the Karl Fischer method, and determining non-volatiles.[160] The VOC content in a solventborne automotive paints is determined by the simulation of VOC loss during baking using laboratory panels.[161]

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50 ASTM D 3828-97. Standard test method for flash point by small scale closed tester.
AS/NZS 2106.0-99. Methods for determination of flash point of flammable liquids (closed cup) - General.
AS/NZS 2106.1-99. Methods for the determination of the flash point of flammable liquids (closed cup) - Abel closed cup method.
51 ASTM D 1310-97. Standard test method for flash point and fire point of liquids by tag open-cup apparatus.
52 ASTM D 56-97a. Standard test method for flash point by tag closed tester.
53 ASTM D 92-97. Standard test method for flash and fire points by Cleveland open cup.
ISO 2592-73. Petroleum product - Determination of flash and fire points - Cleveland open cup method.

SFS-EN 22592. Petroleum products. Determination of flash and fire points. Cleveland open cup method (ISO 2592).

54 ASTM D 93. Standard test methods for flash-point by Pensky-Martens closed cup tester.
AS/NZS 2106.2-99. Methods for determination of the flash point of flammable liquids (closed cup) - Pensky-Martens closed cup method.
DIN EN 22719-93. Petroleum products and lubricants. Determination of flash point. Pensky-Martens closed cup method (ISO 2719).
ISO 2719-88. Petroleum products and lubricants - Determination of flash point - Pensky-Martens closed cup method.
SFS-EN 22719. Petroleum products and lubricants. Determination of flash point. Pensky-Martens closed cup method (ISO 2719)

55 ASTM D 3941-96. Standard test method for flash point by the equilibrium method with a closed-cup apparatus.
AS/NZS 2106.3-99. Methods for the determination of the flash point of flammable liquids (closed cup) - Flash/no flash test - Rapid equilibrium method.
AS/NZS 2106.4-99. Methods for the determination of the flash point of flammable liquids (closed cup) - Determination of flash point - Rapid equilibrium method.
AS/NZS 2106.5-99. Methods for the determination of the flash point of flammable liquids (closed cup) - Flash/no flash test - Closed cup equilibrium method.
AS/NZS 2106.6-99. Methods for the determination of the flash point of flammable liquids (closed cup) - Determination of flash point - Closed cup equilibrium method.
DIN EN 456-91. Paints, varnishes and related products. Determination of flash point. Rapid equilibrium method (ISO 3679).
ISO 1516-81. Paints, varnishes, petroleum and related products - Flash/no flash test - Closed cup equilibrium method.
ISO 1523-83. Paints, varnishes, petroleum and related products - Determination of flash point - Closed cup equilibrium method.
ISO 3679-83. Paints, varnishes, petroleum and related products - Determination of flash point - Rapid equilibrium method.
ISO 3680-83. Paints, varnishes, petroleum and related products - Flash/no flash test - Rapid equilibrium method.
SFS-EN 456-93. Paints, varnishes, and related products. Determination of flash point. Rapid equilibrium method (ISO 3679).

56 ASTM E 502-94. Standard test method for selection and use of ASTM standards for the determination of flash point of chemicals by closed cup methods.

57 ASTM D 1015-94. Standard test method for freezing points of high-purity hydrocarbons.
JIS K 0065-92. Test method for freezing point of chemical products.

58 ASTM D 1016-94. Standard test method for purity of hydrocarbons from freezing points.

59 ASTM D 852-97. Standard test method for solidification point of benzene.
ISO 5278-80. Benzene - Determination of crystallizing point.

60 ASTM D 4755-95. Standard test method for free halogenated organic solvents and their admixtures.

61 ASTM D 2306-96. Standard test method for $C_8$ hydrocarbon analysis by gas chromatography.
DIN 51437-89. Testing of benzene and benzene homologues. Determination of the content of non-aromatics, toluene and C8-aromatics in benzene. Gas chromatography.
DIN 51448-1-97. Testing of liquid petroleum hydrocarbons. Determination of hydrocarbon types. Part 1: Gas chromatographic analysis by column switching procedure.

62 ASTM D 3797-96. Standard test method for analysis of o-xylene by gas chromatography.

63 ASTM D 3798-96b. Standard test method for analysis of p-xylene by gas chromatography.

64 ASTM D 4492-98. Standard test method for analysis of benzene by gas chromatography.

65 ASTM D 3271-87-93. Standard test method for direct injection of solvent reducible paints into a gas chromatograph for solvent analysis.
DIN 55682-94. Solvents for paints and varnishes. Determination of solvents in water-thinnable coating materials. Gas chromatographic method. (Amendment A1 - DIN 55682/A1-98).
DIN 55683-94. Solvents for paints and varnishes. Determination of solvents in coating materials containing organic solvents only. Gas chromatographic method.

66 ASTM D 5830-95. Standard test method for solvent analysis in hazardous waste using gas chromatography.

67 ASTM D 4757-97. Standard practice for placarding solvent vapor degreasers.

68 ASTM D 3844-96. Standard guide for labeling halogenated hydrocarbon solvent containers.
69 ASTM D 1296-96. Standard test method for odor of volatile solvents and diluents.
ISO 2498-74. Methyl ethyl ketone for industrial use - Examination for residual odor.
70 ASTM E 679-97. Standard practice for determination of odor and taste thresholds by forced-choice ascending concentration series method.
71 ASTM E-769-97. Standard test methods for odor of methanol, ethanol, n-propanol, and isopropanol.
72 ASTM E 1075-97. Standard test methods for odor of ethylene glycol, diethylene glycol, triethylene glycol, propylene, glycol, and dipropylene glycol and taste of propylene glycol.
73 ASTM D 4494-95. Standard test method for detecting residual odor of drycleaning grade perchloroethylene.
74 ASTM D 268-96. Standard guide for sampling and testing volatile solvents and chemical intermediates for use in paint and related coatings and material.
75 ASTM D 1353-96. Standard test method for nonvolatile matter in volatile solvents for use in paint, varnish, lacquer, and related products.
76 ASTM D 5286-95. Standard test methods for determination of transfer efficiency under general conditions for spray application of paints.
77 ASTM D 1617-96. Standard test method for ester value of solvents and thinners.
78 ASTM D 2110-96. Standard test method for pH of water extractions of halogenated organic solvents and their admixtures.
79 ASTM D 3545-95. Standard test method for alcohol content and purity of acetate esters by gas chromatography.
DIN 55686-92. Solvents for paints and varnishes. Acetic esters. Gas chromatographic determination of the degree of purity.
80 ASTM D 6229-98. Standard test method for trace benzene in hydrocarbon solvents by capillary gas chromatography.
81 ASTM D 4534-93. Standard test method for benzene content of cyclic products by gas chromatography.
82 ASTM D 435-96. Standard test method for determination of trace thiophene in refined benzene by gas chromatography.
DIN 51438-91. Testing of benzene and benzene homologues. Determination of the content of thiophene in benzene. Gas chromatography.
83 ASTM D 5713-96. Standard test method for analysis of high purity benzene for cyclohexane feedstock by capillary gas chromatography.
84 ASTM D 3447-96. Standard test method for purity of halogenated organic solvents.
CAN/CGSB 1-GP-71 No. 73-96. Methods of testing paints and pigments. Beilstein test for chlorinated solvents.
85 ASTM D 3742-94. Standard test method for 1,1,1-trichloroethane content.
86 ASTM D 2804-98. Standard test method for purity of methyl ethyl ketone by gas chromatography.
87 ASTM D 3329-94a. Standard test method for purity of methyl isobutyl ketone by gas chromatography.
88 ASTM D 3257-97. Standard test methods for aromatics in mineral spirits by gas chromatography.
89 ASTM D 1685-95. Standard test method for traces of thiophene in benzene by spectrophotometry.
90 ASTM D 2031-97. Standard test method for reducing substances in refined pyridine.
91 ASTM D 3961-98. Standard test method for trace quantities of sulfur in liquid aromatic hydrocarbons by oxidative microcoulometry.
ISO 5282-82. Determination of sulfur content - Pitt-Ruprecht reduction and spectrophotometric determination method.
ISO 8754-92. Petroleum products - Determination of sulfur content - Energy-dispersive X-ray fluorescence method.
SFS-EN ISO 8754. Petroleum products. Determination of sulfur content. Energy-dispersive X-ray fluorescence method.
ISO 14596-98. Petroleum products - Determination of sulfur content - Wavelength-dispersive X-ray fluorescence method.
92 ASTM D5194-96. Standard test method for trace chloride in liquid aromatic hydrocarbons.
93 ASTM D 5808-95. Standard test method for determining organic chloride in aromatic hydrocarbons and related chemicals by microcoulometry.
94 ASTM E 299-97. Standard test method for trace amounts of peroxides in organic solvents.
95 ASTM D 4952-97. Standard test method for qualitative analysis for active sulfur species in fuels and solvents (Doctor Test).
ISO 5275-79. Aromatic hydrocarbons - Test for presence of mercaptans (thiols) - Doctor test.

96 ASTM D 2109-96. Standard test methods for nonvolatile matter in halogenated organic solvents and their admixtures.
BS 5598-3-79. Methods of sampling and test for halogenated hydrocarbons. Determination of residue on evaporation (ISO 2210).
ISO 759-81. Volatile organic liquids for industrial use - Determination of dry residue after evaporation on water bath - General method.
ISO 2210-72. Liquid halogenated hydrocarbons for industrial use - Determination of residue on evaporation.
ISO 5277-81. Aromatic hydrocarbons - Determination of residue on evaporation of products having boiling points up to 150°C.
JIS K 0067-92. Test methods for loss and residue of chemical products.
SFS 3773-77. Determination of residue on evaporation.
97 ASTM D 1364-95. Standard test method for water in volatile solvents (Karl Fischer reagent titration method).
BS 2511-70. Methods for determination of water (Karl Fischer method).
ISO 6191-81. Light olefins for industrial use - Determination of traces of water - Karl Fischer method.
ISO 8917-88. Light olefins for industrial use - Determination of water - Guidelines for use of in-line analyzers.
SFS 3774-76. Determination of water by the Karl Fischer method.
98 ASTM D 3401-97. Standard test methods for water in halogenated organic solvents and their admixtures.
99 ASTM D 3443-95. Standard test method for chloride in trichlorotrifluoroethane.
100 ASTM D 3741-95. Standard test methods for appearance of admixtures containing halogenated organic solvents.
101 ASTM D 611-98. Standard test methods for aniline point and mixed aniline point of petroleum products and hydrocarbon solvents.
ISO 2977-97. Petroleum products and hydrocarbon solvents - Determination of aniline point and mixed aniline point.
102 ASTM D 1363-97. Standard test method for permanganate time of acetone and methanol.
ISO 757-7-83. Acetone for industrial use - Methods of test - Part 4: Determination of permanganate time.
103 ASTM D 1612-95. Standard test method for acetone in methanol.
104 ASTM D 1218-96. Standard test method for refractive index and refractive dispersion of hydrocarbon liquids.
ISO 5661-83. Petroleum products - Hydrocarbon liquids - Determination of refractive index.
JIS K 0062-92. Test methods for refractive index of chemical products.
105 ASTM F 151-97. Standard test method for residual solvents in flexible barrier materials.
106 ASTM D 1133-97. Standard test method for Kauri-butanol value of hydrocarbon solvents.
107 ASTM D 2779-97. Standard test method for estimation of solubility of gases in petroleum liquids.
108 ASTM E 1195-93. Standard test method for determining a sorption constant, $K_{oc}$, for an organic chemical in soil and sediments.
109 ASTM D 5404-97. Standard practice for recovery of asphalt from solution using the rotary evaporator.
110 ASTM D 304-95. Standard specification for n-butyl alcohol (butanol).
BS 508-1-86. Butan-1-ol for industrial use. Specification for butan-1-ol.
BS 508-2-84. Butan-1-ol for industrial use. Methods of test.
JIS K 1504-93. Butanol.
JIS K 8810-96. 1-butanol.
111 ASTM D 319-95. Standard specification for amyl alcohol (synthetic).
112 ASTM D 331-95. Standard specification for 2-ethoxyethanol.
113 ASTM D 770-95. Standard specification for isopropyl alcohol.
BS 1595-1-86. Propan-2-ol (isopropyl alcohol) for industrial use. Specification for propan-2-ol (isopropyl alcohol).
BS 1595-2-84. Propan-2-ol (isopropyl alcohol) for industrial use. Methods of test.
ISO 756-1-81. Propan-2-ol for industrial use - Methods of test - Part 1: General.
JIS K 1522-78. Isopropyl alcohol.
114 ASTM D 1007-95. Standard specification for sec-butyl alcohol.
ISO 2496-73. sec-butyl alcohol for industrial use - List of methods of test.
JIS K 1523-78. 2-butanol.
115 ASTM D 1152-97. Standard specification for methanol (methyl alcohol).
BS 506-1-87. Methanol for industrial use. Specification for methanol.

BS 506-2.84. Methanol for industrial use Methods of test.
CAN/CGSB-3-GP-531M. Methanol, technical.
ISO 1387-82. Methanol for industrial use - Methods of test.
JIS K 1501-93. Methanol.
116 ASTM D 1719-95. Standard specification for isobutyl alcohol.
117 ASTM D 1969-96. Standard specification for 2-ethylhexanol (synthetic).
BS 1835-91. Specification for 2-ethylhexan-1-ol for industrial use.
118 ASTM D 2627-97. Standard specification for diacetone alcohol.
BS 549-70. Specification for diacetone alcohol.
ISO 2517-74. Diacetone alcohol for industrial use - List of methods of test.
119 ASTM D 3128-97. Standard specification for 2-methoxyethanol.
120 ASTM D 3622-95. Standard specification for 1-propanol (n-propyl alcohol).
JIS K 8838-95. 1-propanol.
121 ASTM D 841-97. Standard specification for nitration grade toluene.
AS 3529-1988. Solvent - Toluene.
ISO 5272-79. Toluene for industrial use - Specification.
JIS K 8680-96. Toluene.
122 ASTM D 843-97. Standard specification for nitration grade xylene.
ISO 5280-79. Xylene for industrial use - specification.
JIS K 8271-96. Xylene.
123 ASTM D 4734-98. Standard specification for refined benzene-545.
124 ASTM D 5136-96. Standard specification for high purity p-xylene.
125 ASTM 5211-97. Standard specification for xylenes for p-xylene feedstock.
126 ASTM D 5471-97. Standard specification for o-xylene 980.
127 ASTM D 5871-98. Standard specification for benzene for cyclohexane feedstock.
ISO 5721-79. Benzene for industrial use - Specification.
128 ASTM D 3734-96. Standard specification for high-flash aromatic naphthas.
129 ASTM D 5309-97. Standard specification for cyclohexane 999.
130 ASTM D 235-95. Standard specification for mineral spirits (petroleum spirits) (hydrocarbon dry cleaning solvent).
AS 35-1988. Solvents - Mineral turpentine and white spirit.
BS 245-76. Specification for mineral solvents (white spirit and related hydrocarbon solvents) for paints and other purposes.
CAN/CGSB-53.7-92. Duplicating liquid, Direct process, Spirit type.
131 ASTM D 1836-94. Standard specification for commercial hexanes.
132 ASTM D 3735-96. Standard specification for VM&P naphthas.
133 ASTM D 740-97. Standard specification for methyl ethyl ketone.
134 ASTM D 4360-90. Standard specification for n-amyl ketone.
135 ASTM D 329-95. Standard specification for acetone.
BS 509-1-87. Acetone for industrial use. Specification for acetone.
BS 509-2-84. Acetone for industrial use. Methods of test.
CAN/CGSB-15.50-2. Technical grade acetone.
ISO 757-1-82. Acetone for industrial use - Methods of test - Part 1: General.
JIS K 1503-59. Acetone.
JIS K 8035-95. Acetone.
SFS 3775 ISO/R 757. Acetone for industrial use. Methods of test.
136 ASTM D 740-94. Standard specification for methyl ethyl ketone.
CAN/CGSB-15.52-92. Technical grade methyl ethyl ketone.
ISO 2597-73. Methyl ethyl ketone for industrial use - List of methods of test.
JIS K 1524-78. Methyl ethyl ketone.
137 ASTM D 1153-94. Standard specification for methyl isobutyl ketone.
ISO 2499-74. Methyl isobutyl ketone for industrial use - List of methods of test.
138 ASTM D 2917-94. Standard specification for methyl isoamyl ketone.
ISO 2500-74. Ethyl isoamyl ketone for industrial use - List of methods of test.
139 ASTM D 5137-95. Standard specification for hexyl acetate.
140 ASTM D 1718-94. Standard specification for isobutyl acetate (95% grade).
BS 551-90. Specification for butyl acetate for industrial use.
141 ASTM D 2634-98. Standard specification for methyl amyl acetate (95% grade).

142 ASTM D 3130-95. Standard specification for n-propyl acetate (96% grade).
143 ASTM D 3131-97. Standard specification for isopropyl acetate (99% grade).
BS1834-68. Specification for isopropyl acetate.
144 ASTM D 3540-98. Standard specification for primary amyl acetate, synthetic (98% grade).
145 ASTM D 3728-97. Standard specification for 2-ethoxyethyl acetate (99% grade).
146 ASTM D 5164-96. Standard specification for propylene glycol and dipropylene glycol.
JIS K 1530-78. Propylene glycol.
147 ASTM D 4701-95. Standard specification for technical grade methylene chloride.
ISO 1869-77. Methylene chloride for industrial use - List of methods of test.
JIS K 1516-84. Methyl chloride.
148 ASTM D 4079-95. Standard specification for vapor-degreasing grade methylene chloride.
149 ASTM D 4080-96. Standard specification for trichloroethylene, technical and vapor-degreasing grade.
ISO 2212-72. Trichloroethylene for industrial use - Methods of test.
JIS K 1508-82. Trichloroethylene.
150 ASTM D 4081-95. Standard specification for drycleaning-grade perchloroethylene.
ISO 2213-72. Perchloroethylene for industrial use - Methods of test.
JIS K 1521-82. Perchloroethylene.
151 ASTM D 4126-95. Standard specification for vapor-degreasing grade and general solvent grade 1,1,1-trichloroethane.
AS 2871-1988. Solvents. 1,1,1-trichloroethane (inhibited).
ISO 2755-73. 1,1,1-trichloroethane for industrial use - List of methods of test.
JIS K 1600-81. 1,1,1-trichloroethane.
152 ASTM D 4376-94. Standard specification for vapor-degreasing grade perchloroethylene.
153 ASTM D 4206-96. Standard test method for sustained burning of liquid mixtures using small scale open-cup apparatus.
ISO/TR 9038-91. Paints and varnishes - Determination of the ability of liquid paints to sustain combustion.
154 ASTM E 1194-93. Standard test method for vapor pressure.
DIN EN 12-93. Petroleum products. Determination of Reid vapor pressure. Wet method.
ISO 3007-99. Petroleum products and crude petroleum - Determination of vapor pressure - Reid method.
155 ASTM D 2879-96. Standard test method for vapor pressure-temperature relationship and initial decomposition temperature of liquids by isoteniscope.
156 ASTM D 4486-96. Standard test method for kinematic viscosity of volatile and reactive liquids.
DIN EN ISO 3104. Petroleum products. Transparent and opaque liquids. Determination of kinematic viscosity and calculation of dynamic viscosity.
ISO 3104-94. Petroleum products - Transparent and opaque liquids - Determination of kinematic viscosity and calculation of dynamic viscosity.
SFS-EN ISO 3104. Petroleum products. Transparent and opaque liquids. Determination of kinematic viscosity and calculation of dynamic viscosity.
157 ASTM D 88-94. Standard test method for Saybolt viscosity.
158 ASTM D 1545-93. Standard test method for viscosity of transparent liquids by bubble time method.
159 ASTM D 5201-97. Standard practice for calculating formulation physical constants of paints and coatings.
ISO/FDIS 11890-1-99. Paints and varnishes - Determination of volatile organic compound content (VOC) - Part 1: Difference method.
ISO/FDIS 11890-2-99. Paints and varnishes - Determination of volatile organic compound (VOC) content - Part 2: Gas-chromatographic method.
160 ASTM D 5325-97. Standard test method for determination of weight percent volatile content of water-borne aerosol paints.
161 ASTM D 5087-94. Standard test method for determining amount of volatile organic compound (VOC) released from solventborne automotive coatings and available for removal in a VOC control device (abatement).

# 15.2 SPECIAL METHODS OF SOLVENT ANALYSIS

## 15.2.1 USE OF BREATH MONITORING TO ASSESS EXPOSURES TO VOLATILE ORGANIC SOLVENTS

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### 15.2.1.1 Principles of breath monitoring

Exposure to organic solvents may occur as a result of occupation, diet, lifestyles, hobbies, etc., in a variety of environments (occupational, residential, ambient). Solvents reach the organism through inhalation, ingestion and dermal exposure. The magnitude of exposures, however, may be modulated by factors such as the use of protective equipment (decrease) or physical exertion (increase). All sources and routes of exposure are integrated in the resulting internal dose. The dose at the target organ may trigger mechanisms that eventually may result in irreversible biological changes and disease. Cellular repair mechanisms and individual susceptibility have a significant effect on the onset of disease, resulting in a broad distribution of outcomes. This continuum between exposure to organic solvents and disease can be depicted, in a simplified way, in Figure 15.2.1.1. Biological monitoring focuses on the elucidation of the first step of this process; i.e., the relationship between environmental exposure and internal dose. Whereas the dose at the target organ is the biologically important, concentrations of the solvent (or its metabolites) may be more easily measured in other fluid or tissue samples, such as blood, breath, urine, etc.

Traditional workplace monitoring relied on measurements of airborne contaminant concentrations at the breathing zone, and comparisons to reference values or regulatory benchmarks, such as the Threshold Limit Values (TLVs) and the Permissible Exposure Limits (PELs) in the USA, and similar values promulgated by the European Union and other organizations. Air measurements, however, may not always represent actual personal exposures since workers may be exposed dermally and orally, or they may be using protective equipment. Over the last decade, work-place monitoring programs have expanded to include biological monitoring. Biological monitoring gives an estimate of the dose inside the body (or specific target organ) rather than the concentration of the solvent in the external environment. Internal dose is defined as the amount of the solvent taken up by the whole organism (or a body compartment) over a specified period of time.[1] Depending on the chemical, the time of sampling and the tissue analyzed, the dose may reflect the amount of the chemical recently absorbed, the amount of the chemical stored (body burden), or the amount of the chemical bound to the active sites of a receptor. It is the dose at the receptor or sensitive tissue (the biologically effec-

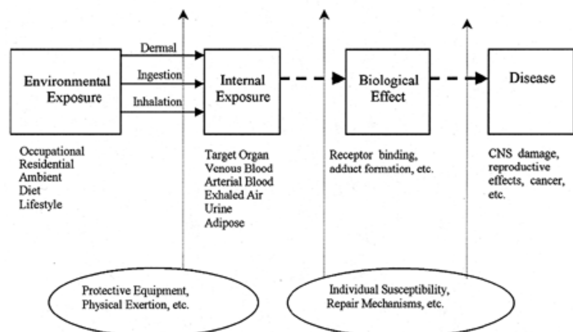


Figure 15.2.1.1. Relationship between exposure to volatile organic solvents and potential outcomes.

tive dose) that is related to toxicity and/or damage. In general, the dose at the receptor can not be measured because, at this time, available methodology is unacceptably invasive to be employed. On the other hand, biological monitoring allows measurements to be made on body fluids or tissues that would serve as surrogate measurements of the dose at the receptor. Certain conditions, however, are required for meaningful interpretations of the analytical results.

1. There should be adequate information on the absorption, distribution, biotransformation, metabolism and elimination of the chemical in the body. This type of information will indicate what tissues to sample, for what compound (parent chemical or a metabolite) and at what time. The latter issue is very important with chemicals that are eliminated rapidly (i.e., those with short half-lives).

2. The concentration of the chemical (or its metabolite) to be measured in the selected medium (breath, blood, urine) must be in equilibrium with the concentration of the chemical at the target organ.

3. The assay has to be sensitive, in order to detect low levels of the compound before any adverse effects take place; specific, in order to link exposure to dose; and accurate and precise, in order to be reliable.

Very few chemicals have been adequately studied to allow biological monitoring according to the above criteria. This is, however, an expanding area, as indicated by the number of substances for which Biological Exposure Indices (BEIs) have been proposed by the American Conference of Governmental Industrial Hygienists (ACGIH). In the 1984-85 Threshold Limit Value (TLV) booklet there were BEIs for 10 chemicals; in the 1998 booklet there were 37 compounds with adopted BEIs.[2] BEIs are considered reference values intended as guidelines and do not determine whether the worker is at risk of disease. They are supplementary to TLVs in evaluating workers' exposures to workplace hazardous agents. The recommended values of BEIs are based on data correlating exposure intensity and biological effects from field studies and/or on pharmacokinetic treatment of data obtained from controlled human exposures. The BEIs developed by the ACGIH, assume 8-hour exposures for 5 days, followed by 2 days of non-exposure, and they strictly specify the time of sample collection.

A clear advantage of biological monitoring over environmental or personal monitoring is that the estimate of the internal dose takes into account all possible routes of entry (inhalation, ingestion and dermal exposure). In addition, exposures other than occupational, such as through diet, hobbies or residential air, all contribute to the internal dose, which is related to adverse effects. When the effect of occupational exposure alone needs to be evaluated, non-occupational exposures need to be assessed and controlled to allow interpretation of biological monitoring results. A special case is the evaluation of protective equipment in reducing workplace exposures.

Biological monitoring is limited by parameters that can affect the exposure and dose relationship. Such parameters include the following: biological conditions (age, sex, obesity, pregnancy, disease), physical workload, or exposure to other agents (other chemicals, tobacco smoking, dietary components) that in some way interfere with the uptake, distribution, metabolism or elimination of the chemical in the body.[3-6] Such parameters may introduce large inter-individual variation in the resulting dose. When simulation techniques were used to evaluate the influence of these parameters, it was shown[6] that up to 100% bias may result from inter-individual variation in the metabolism of aromatic compounds. If these differences occur randomly, they will widen the confidence limits of the biological monitoring results, decreasing the statistical power of a study. This is just one example illustrating the care that must be taken when interpreting biological monitoring results.

In short, biological monitoring should be used with specific objectives, such as to evaluate exceedence of a reference or regulatory value; dermal or oral exposure not assessed by personal monitoring; efficacy of protective equipment; etc. Accordingly, current, recent or cumulative exposure may need to be assessed and, therefore, the appropriate chemical (parent or metabolite) should be monitored in the appropriate specimen, collected at the appropriate time, from the appropriate worker(s).

### 15.2.1.2 Types of samples used for biological monitoring

Of the various tissues/fluids that can be used for biological monitoring, the most common are blood, urine and exhaled air because they are relatively easy to obtain.

Blood collection is the most invasive of the three and the chemical analysis may be subject to interferences because of the complex matrix. Additionally, concern over hepatitis and HIV infection, and confidentiality issues, make blood sampling less attractive for routine collection. Nevertheless, concentrations of a compound of interest in blood are easier to relate to concentrations at the target organ.

Analysis of urine samples allows elimination rates of the chemical or its metabolite(s) to be ascertained. However, measurements in urine require specimen collection under a schedule (first morning urine, or 24-hour sample) that can create logistical difficulties. The concentration of the contaminant, or its metabolite(s), is usually corrected for the dilution of the urine (specific gravity or, more commonly, creatinine correction) to be used as a measure of dose.

Collection of breath samples is the easiest of the three methods. It is non-invasive; it can take place almost anywhere; and the analytical matrix is usually very simple. The basic assumption underlying breath monitoring is the existence of gaseous equilibrium between the concentration of the vapor of interest in the alveolar air and in the arterial blood. This relationship must be established for every solvent for which biological monitoring by exhaled air analysis is considered. The apparent simplicity of breath sampling should not be overstated, however, and the limitations and pitfalls should be identified before any large scale, routine applications of breath monitoring can be envisioned. Since breath is a non-homogeneous mixture of air coming from different regions of the lung with varying ventilation, perfusion, and diffusion characteristics, some portion of the breath should be consistently identified and tested for its relationship to the blood concentration.

The ACGIH only recommends measurements in exhaled air when:[7]

- The chemical is poorly metabolized and, therefore, exhalation is the primary route of elimination;
- Sampling can occur when the effect of time and other circumstantial factors can be controlled;
- The sampling method is well defined.

### 15.2.1.3 Fundamentals of respiratory physiology

In order for any inhaled solvent vapors to enter the blood circulation, they need to reach the alveoli, cross the gas-blood interface and dissolve in the blood. Gas is carried to the gas-blood interface by airways, while blood is carried by blood vessels. Since these functions take place in the lung, the essentials of respiratory physiology will be briefly reviewed.

The primary function of the lungs is to allow oxygen to move from the inhaled air into the arterial blood and to allow carbon dioxide to move from the venous blood to the exhaled air. Both oxygen and carbon dioxide move between air and blood by simple diffusion from an area of high partial pressure to an area of low partial pressure. The barrier between air and blood is less than 0.5 μm in thickness and has an area of between 50 to 100 $m^2$.[8] This barrier is very efficient for gas exchange by molecular diffusion, according to Fick's 2nd law.

The airways consist of a series of tubes that become narrower and shorter as they extend deeper into the lung. The first segment, the trachea, divides into two main bronchi, which in turn divide into lobar and segmental bronchi, which turn into terminal bronchioles. All these segments collectively constitute the anatomical dead space with a volume of about 150 mL for an adult male. The function of this conducting portion of the lung is to transport the inspired air into the gas exchange regions. The terminal bronchioles divide within a distance of 5 mm into respiratory bronchioles and alveolar ducts, which are completely lined with alveoli. This region of the lung is the respiratory zone where the gas exchange takes place. The volume of this zone (about 2,500 mL) makes up most of the lung volume.

#### *15.2.1.3.1 Ventilation*

Air is drawn into the lungs by contractions of the diaphragm and the intercostal muscles, which raise the rib cage, and flows to the terminal bronchioles by bulk flow. After that point, the velocity of the inspired air diminishes as the cross sectional area of the airways increases dramatically. Thereafter, ventilation is carried out by molecular diffusion, which results in rapid exchange of gases.

Similarly to the airways, the pulmonary blood vessels form a series of branching tubes from the pulmonary artery to the capillaries and back to the pulmonary veins. The diameter of a capillary segment is about 10 μm, just large enough for a red blood cell. The capillaries form a dense network in the walls of the alveoli, with the individual capillary segments so short that the blood forms an almost continuous sheet around the alveoli, providing ideal conditions for gas exchange. In about one second, each red blood cell transverses two or three alveoli in the capillary network, achieving complete equilibration of oxygen and carbon dioxide between alveolar gas and capillary blood.

For an adult male at rest, a typical breathing frequency is 15 breaths per minute and a typical exhaled air volume (tidal volume), at rest, is about 500 mL. The total volume of air exhaled in 1 min is, therefore, about 7,500 mL. This is the minute volume total ventilation. The inhaled volume during the same time is slightly greater, since more oxygen is taken up than carbon dioxide released. Not all the inhaled air reaches the alveoli; the anatomical dead space (volume of conducting airways where no gas exchange takes place) contains approximately 150 mL of each breath. Therefore, the volume of fresh inhaled air that reaches the alveoli in 1 min is 5,250 mL [(500-150) mL/breath * 15 breaths/min = 5,250 mL/min].[8] This is the alveolar ventilation and it represents the volume of fresh air available for gas exchange in 1 min. The alveolar ventilation cannot be measured directly, but it can be calculated by measuring the minute volume and subtracting the anatomical dead space. The former is easily measured by collecting all the expired air in a bag. The volume of the anatomical dead space can be assumed to be 150 mL, with minimal variation for all adults, and the dead space ventilation can be calculated given a respiratory frequency. Subtraction of the dead space ventilation from the total ventilation (or minute volume) results in the alveolar ventilation. Pulmonary ventilation may be affected by chronic diseases such as asthma or pulmonary fibrosis, or even by transient hyperventilation or hypoventilation.

#### *15.2.1.3.2 Partition coefficients*

Whereas the rate of transfer of a solvent vapor between alveolar air and capillary blood is determined by its diffusivity, the equilibrium between these matrices is determined by the blood/air partition coefficient (λ). This is the ratio of the concentration of the vapor in blood and air at 37°C, at equilibrium. Partition coefficients are commonly determined in vitro, and occasionally in vivo.[2,9,10] Although considered constant at a particular temperature, partition coefficients may be affected by the composition of the blood. The blood/air partition coeffi-

cients of some industrial solvents were shown to increase by up to 60% after meals,[4] presumably due to an increase in lipid content of the blood.

Depending on the affinity of a gas or vapor for blood, the transfer from the alveoli to the capillaries may be diffusion-limited or perfusion-limited. The difference can be illustrated by examining two solvents, styrene and methyl chloroform. Because of the high solubility of styrene ($\lambda$=52)[11] large amounts of it can be taken up by the blood, and the transfer is only diffusion-limited. On the other hand, methyl chloroform is not very soluble ($\lambda$=1.4)[12] and its partial pressure rises rapidly to that of the alveolar air, at which point no net transfer takes place. The amount of methyl chloroform taken up by the blood will depend exclusively on the amount of available blood flow and not on the diffusion properties of the gas-blood interface. This kind of transfer is perfusion-limited.

It is the concentration in the mixed venous blood, however, that better reflects the concentration at the target organ. It has been shown that:[13]

$$P / Pv = \frac{\lambda}{\lambda + V / Q}$$

where:

| | |
|---|---|
| P | partial pressure in the alveoli and in the arterial blood leaving that region of the lung |
| Pv | partial pressure in the venous blood coming to the lung |
| $\lambda$ | blood/air partition coefficient |
| V | alveolar ventilation |
| Q | blood flow of the lung region under consideration |

The greater the blood/air partition coefficient, the closer the arterial concentration will be to the venous concentration.

The concentration in the alveolar air will reflect both arterial and venous blood if the blood/air partition coefficient of the vapor is greater than 5,[14] a criterion that applies to most industrial solvents. In a normal subject at rest, the ventilation-to-perfusion ratio (V/Q) ranges from 0.7 to 1.0, with an average of about 0.9.[15] Variations in the V/Q ratio exist in various parts of the lung because not all alveoli are ventilated and perfused in ideal proportions. The V/Q ratio may vary from 0.5 at the lung base to 3.0 at the apex,[16] and it becomes more homogeneous with physical exertion.[17] When the partition coefficient exceeds 10, however, even large degrees of ventilation/perfusion imbalance have very little effect on the relationship between alveolar and mixed venous solvent partial pressures.[14]

### *15.2.1.3.3 Gas exchange*

When a person is exposed to a volatile organic solvent through inhalation, the solvent vapor diffuses very rapidly through the alveolar membranes, the connective tissues and the capillary endothelium and into the red blood cells or plasma. With respiratory gases the whole process takes less than 0.3 seconds.[15] This results in almost instantaneous equilibration between the concentration in alveolar air and in blood and, therefore, the ratio of the solvent concentration in pulmonary blood to that in alveolar air should be approximately equal to the partition coefficient. As the exposure continues, the solvent concentration in the arterial blood exceeds that in the mixed venous blood.[18] The partial pressures in alveolar air, arterial blood, venous blood and body tissues reach equilibrium at steady state. When the exposure stops, any unmetabolized solvent vapors are removed from the systemic circulation through pulmonary clearance. During that period the concentration in the arterial blood is lower than in the mixed venous blood[18] and the solvent concentration in alveolar air will depend on the pulmonary ventilation, the blood flow, the solubility in blood and the concentration in the

mixed venous blood. These are the prevailing conditions when breath monitoring takes place after the end of the exposure[17] and therefore, samples of alveolar air after exposure should reflect concentrations in mixed venous blood. During exposure, however, alveolar air should reflect concentrations in arterial blood. Solvent concentrations in arterial and venous blood diverge even more with physical exertion.

To summarize, the uptake of solvent vapors through inhalation will depend on the following factors:[3]

1. Pulmonary ventilation, i.e., the rate at which fresh air (and solvent vapor) enters the lungs. This is determined by the metabolic rate and therefore depends on physical exertion. The concentration in alveolar air approaches that in inhaled air when the physical exertion is great. When exertion is low, the alveolar air concentration approaches the concentration in mixed venous blood.

2. Diffusion of the solvent vapor through the gas-blood interface. According to Fick's 2nd law, the rate of diffusion through the interface depends on the concentration gradient across the tissue membrane. Therefore, the concentration of the vapor leaving the lungs depends on the amount of vapor entering the alveoli, on the solubility in blood (blood/gas partition coefficient) and on the flow rate of the blood through the lungs. For lipophilic chemicals, such as organic solvents that readily cross cellular membranes, diffusion is not the rate-limiting factor.[18]

3. The solubility of the vapor in the blood. The higher the blood/gas partition coefficient, the more rapidly the vapor will diffuse into the blood, until equilibrium is achieved. At equilibrium the net diffusion between blood and air ceases, but the concentrations in air and blood may still be different. A highly soluble vapor will therefore demonstrate a lower alveolar air concentration relative to the inhaled air concentration during exposure, and a higher alveolar concentration relative to the inhaled air concentration after exposure.

4. The circulation of the blood through the lungs and tissues. This depends on the cardiac output and therefore, on physical exertion. For a soluble vapor, high exertion moves blood faster, increasing uptake.

5. The diffusion of the vapor through the tissue membranes, which depends on membrane permeability and is governed by Fick's law, as discussed in #2 above.

6. The solubility of the solvent vapor in the tissue. This reflects the tissue/blood partition coefficient of the solvent vapor and depends largely on the lipid composition of the particular tissue. Lipophilic vapors will exhibit high solubility in tissues with high fat content, freeing blood of vapors and increasing uptake.

Substances that are not well metabolized are eliminated primarily through the lungs[3] and to a lesser extent through the kidneys. During elimination through the lungs the same physiological principles apply in a reverse sequence.

#### *15.2.1.4 Types of exhaled air samples*

Samples obtained from even a single breath may contain different concentrations of the solvent vapor of interest depending on the way the sample was collected. This is a result of the non-homogeneity of the exhaled air discussed previously. Figure 15.2.1.2 depicts a concentration curve of a solvent in exhaled air. When measurement takes place in an environment free of that solvent, the first part of the exhalation, corresponding to air in the anatomical dead space, will be solvent-free (Phase 1). Then the solvent concentration curve rises sharply reflecting exhalation of air from the respiratory zone of the lungs (Phase 2). This phase is followed by a slowly increasing plateau, indicating an alveolar steady state (Phase 3). The level of this plateau depends on the preceding ventilatory state of the subject, being higher for a hypoventilating subject and lower for a hyperventilating subject.[6] Sample col-

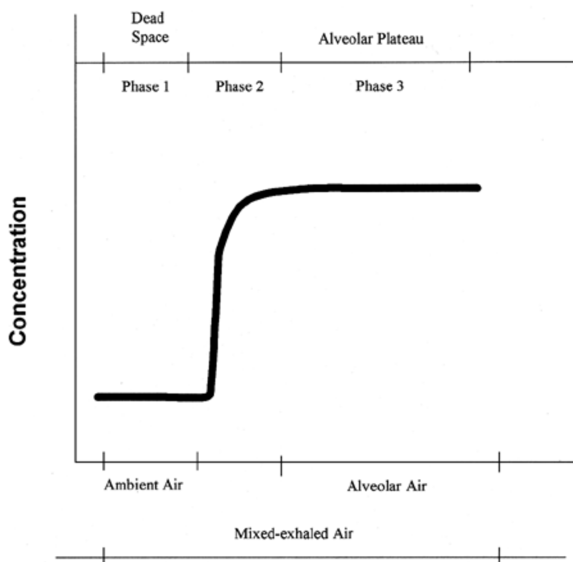


Figure 15.2.2. Solvent concentration in breath during exhalation.

lection at the alveolar plateau constitutes end-exhaled air. When the total expiration is collected (combination of all three phases) the sample is considered mixed-exhaled air. Simultaneous monitoring of $CO_2$,[6,19-21] or oxygen and nitrogen[22] has been suggested to normalize the measured solvent vapor concentrations in breath samples in order to facilitate data interpretation and comparisons.

The various sampling techniques must be evaluated and the one most applicable to the purpose of sampling chosen. The most commonly used sampling techniques based on physiological principles include mixed-exhaled air and end-exhaled air:

Mixed-exhaled air. This technique involves the collection of the entire volume of exhaled air. It corresponds to a mixture of the alveolar air with air from the dead space. The collection apparatus may also contribute to the dead space. Total dead space should be considered and the concentration adjusted, either by subtraction, or by regression against some other technique unaffected by the dead space. Timing of the breath collection is important here since the concentration of the air in the dead space may equal that of the air in the workroom if the sample is taken during exposure, or it may equal zero if taken after the end of exposure.

End-exhaled air. This technique excludes air from the dead space and collects only the last part of the breath, in order to estimate the concentration in the alveolar air which is in equilibrium with the arterial blood. Use of a Haldane-Priestley tube,[23] or simultaneous monitoring of the peaking of the temperature of the breath[19] or its $CO_2$ concentration,[6,19,24] will assure a valid alveolar air sample.[6,25,26]

Two less frequently used techniques include breath holding or rebreathing to homogenize the breath sample:

Breath holding. For compounds that achieve equilibrium slowly, some researchers have used the breath holding technique, which involves holding the breath for 5-30 seconds prior to exhaling into the collection device. This results in a more constant concentration in the exhaled breath. The extent to which the lungs are filled with air (as with a deep inhalation) will affect the results. Kelman[14] showed, however, that breath holding is not necessary for solvents with blood/gas partition coefficients greater than 10.

Rebreathing. With this technique the subject rebreathes his/her exhaled air to provide a more homogenized breath sample. This technique is applicable to solvents that reach steady state very slowly. For prolonged rebreathing, removal of $CO_2$ to avoid pH changes and/or supply of oxygen may be necessary.[27]

### *15.2.1.5 Breath sampling methodology*

The theoretical principles discussed above lead to two choices for collecting breath samples: Should a mixed-exhaled air or an end-exhaled air sample be collected?

End-exhaled air is becoming the standard for breath monitoring. Of the 36 chemicals with established BEIs,[7] end-exhaled air is recommended for 6, while earlier BEIs recommending mixed-exhaled air have been withdrawn. Beyond the regulatory arena, however, mixed-exhaled air continues to be used with satisfactory results.

Methodologically, the difference between these two types of samples is the control over which portion of the breath is collected. Many techniques[28-32] make use of the Haldane-Prestley tube to collect the last portion of the expiration. Earlier work[6,19] used the simultaneous monitoring of $CO_2$ or temperature in the exhaled breath to identify the moment ($CO_2$ or temperature reaching a maximum) when air from the anatomical dead space has been purged and alveolar air can be sampled.

A variety of sampling techniques has been tried for measuring organic vapors in exhaled air. Table 15.2.1.1 shows a classification of these techniques. The main distinction is between direct reading instruments and transportable samples that require analysis in the laboratory. The former group covers the widest range, from simple detector tubes to High Resolution Mass Spectrometry.

**Table 15.2.1.1. Techniques for sampling organic vapors in exhaled air**

| Technique | References |
|---|---|
| A. SAMPLE COLLECTION | |
| A.1 END-EXHALED AIR | |
| Use of Haldane-Prestley tube | 23, 25, 26, 28, 29, 31 |
| Simultaneous $CO_2$ monitoring | 6, 20, 21, 24 |
| Simultaneous temperature monitoring | 19 |
| B. SAMPLE ANALYSIS | |
| B.1 DIRECT READING INSTRUMENTS | |
| B.1.1 DETECTOR TUBES | |
| Colorimetric | 33 |
| B.1.2 GAS CHROMATOGRAPHS with various detectors and modified injector/inlet with mouthpiece | |
| GC/FID | 52 |
| GC/PID | 25, 26 |
| GC/MS | 24, 41, 42, 43 |
| B.2 TRANSPORTABLE SAMPLE COLLECTORS | |
| B.2.1 CONTAINERS | |
| B.2.1.1 Rigid containers | |
| Stainless steel tubes | 24 |
| Glass tubes | 9, 53, 54, 55, 61, 64 |
| Aluminum tubes | 28, 29, 47 |
| B.2.1.2 Flexible containers | |
| Tedlar bags | 6, 49 |
| Saran bags | 54, 55, 60 |
| Mylar bags | |
| B.2.2 ADSORBENTS | |
| B.2.2.1 Tubes/cartridges containing: | |
| Charcoal | 25, 26, 28, 29, 40 |
| Tenax | 31, 49 |
| Silica gel | 6 |
| B.2.2.2 Felt/cloth | 48 |

Specifically, detector tubes operating on colorimetric reaction principles have been used to determine the concentration of alcohol in breath for traffic safety.[33] Alcohol measurements in breath have been thoroughly studied and scrutinized because of the forensic

and legal repercussions associated with the results. Portable devices have been developed for field use with on-site analysis of alcohol.[34-36] These portable devices could be adapted for use with other organic solvents of interest in industrial hygiene and occupational health.

Gas chromatographs (GCs) equipped with suitable detectors allow analysis of more than one solvent vapor at a time. The subject exhales through a valve system and the exhaled volume of air, or a fraction of it, is drawn into the GC.[21,26,37-39] Depending on the detector used, one or more cylinders containing appropriate gases under pressure are required to operate the instrument, limiting its field use. An Electron Capture Detector (ECD), which is the most sensitive for polyhalogenated solvents, requires helium and/or nitrogen. A Flame Ionization Detector (FID), which is a universal detector for aliphatic and aromatic hydrocarbons, requires nitrogen, air and hydrogen. The advent of the Photoionization Detector (PID) offered a compromise, in that it can detect all the above classes of organic solvents (with varying sensitivity) requiring only one gas (nitrogen, air or helium) for its operation. A simple system employed a portable GC/PID modified to sample 1 mL of air from a Haldane-Priestley tube into which the subject would exhale.[25,40] The latter assured collection of end-exhaled air and this device was successfully used to assess exposures to styrene[26] and PERC.[40]

Mass spectrometry (MS) offers the advantages of high sensitivity and specificity at the cost of portability and the need of highly trained operators. Direct exhalation into the MS is not possible because the ion source of the detector operates under high vacuum. Two approaches have been taken to overcome this limitation. In the first, an inlet system was designed where the high pressure breath sample was directed over a methyl silicone membrane, permeable to organic molecules but not to air, that served as the entry port to the ion source operating under high vacuum.[41] With the second approach, the inlet system was attached to the source of an Atmospheric Pressure Ionization mass spectrometer (API/MS), that operates under atmospheric pressure.[42,43]

In summary, the basic advantage of the direct reading/analyzing instruments is that there is no sample collection, transportation, storage, handling and analysis where the sample integrity may be compromised. The disadvantages are the need for expensive, specialized equipment, usually requiring highly trained operators and the difficulty, or some times impossibility, of field use.

The other category of breath sampling methodology involves sample collection in the field with subsequent analysis in the laboratory. There are two groups of devices in this category:

1. Sample containers that hold the total volume or part of the exhalation in a gaseous form.
2. Sample adsorbents that trap the chemicals of interest from the exhaled breath.

In the first group belong rigid containers such as stainless steel, aluminum, or glass vials of various sizes, open at both ends. The subject exhales into the vial and then the two openings are closed, either with caps or with stopcocks. Vials of this kind have been used extensively in experimental studies involving exposures to industrial chemicals.[24,28,29,44-47,53-55,61,64]

The advantages of these containers are inertness of the walls, low cost, reusability and simplicity of the technique. The disadvantages are the potential for contamination, photodegradation, or dilution of the collected breath sample with atmospheric air, sorption and/or reaction of the vapor with surfaces and breakage during transportation or storage. The problem of condensation of water vapor has been addressed by heating the tubes,[28,29,47] but this approach increases the complexity and counters the main advantages. Another limitation is that only a small volume of sample can be withdrawn for analysis from a rigid container, with ensuing restrictions on the analytical sensitivity.

The last limitation is overcome when collapsible bags are used for sample collection. The bags are commonly made of Tedlar (clear) or Mylar (aluminized) material. The subject exhales directly into the bag through a valve and when an appropriate volume has been collected, the valve is closed. The bag is transported to the laboratory where an aliquot is withdrawn for analysis through a septum. The collapsibility of the bag allows any volume to be withdrawn without air infiltration. Disadvantages, however, include losses because of permeation through the walls, sorption and/or reaction of the vapor with the wall surfaces and photodegradation. The latter concern is eliminated with the aluminized Mylar bags, which also minimize sorption and permeation through the walls.

The second group of field sampling devices with subsequent analysis involves the use of adsorbents. Extensively used in personal monitoring in industrial hygiene surveys, these devices were naturally considered for exhaled breath sampling. Commercially available glass tubes containing granular adsorbent material such as activated charcoal, Tenax, molecular sieve, etc. could be used with selected organic vapors. However, because of the high pressure drop during exhalation through the adsorbent bed, and the condensation of water vapor, these materials have not been used much for direct sampling of exhaled breath. One device[48] is, basically, a respirator mask with two commercial charcoal inhalation canisters and an exhalation port fitted with a wafer of charcoal cloth. A Wright respirometer connected to the exhalation port records the volume of exhaled air. Another hand-held device employed a custom-made glass tube containing charcoal to collect organic vapors in exhaled air, while the volume collected was measured with a Wright respirometer and adjusted for back-pressure measured with an attached pressure gauge.[25,40]

Commercial tubes or specially made cartridges containing the appropriate adsorbent have been used in conjunction with bag sampling for exhaled breath monitoring.[49] The subject exhales into the bag, the contents of which are subsequently pumped through the tube or canister containing the adsorbent. This combination offers the advantages of bag sampling (low cost, low resistance), while eliminating the disadvantages (losses, bulkiness). The organic vapors can be thermally desorbed from the adsorbent material and the concentrations measured with polymer-coated surface-acoustic-wave detector,[50] GC or GC/MS analysis.[31,49] Alternatively, solvent vapors can be extracted from the adsorbent with an appropriate solvent prior to conventional analysis.

#### *15.2.1.6 When is breath monitoring appropriate?*

Breath monitoring may be used to assess exposures to solvents either qualitatively or quantitatively. Qualitative information may be useful when exposure to a number of solvents is suspected. For qualitative purposes, either mixed-exhaled or end-exhaled air samples may be used. Provided that the sample collection and the analytical techniques are appropriate, a list of solvents may be thus identified and quantitative measurements planned.

Quantitative information is needed for regulatory enforcement situations, where appropriately collected breath samples are compared to BEIs to assess whether exposure limits have been exceeded. BEIs have been developed after thorough studies of pharmacokinetics for a limited number of occupational contaminants. Exhaled air is used as a surrogate for blood when the relationships between exhaled air, alveolar air and (arterial or mixed venous) blood have been established.

Besides regulatory purposes, breath monitoring can provide valid information on solvent exposures when:

- The pharmacokinetics (absorption, distribution, metabolism, elimination) of the solvent are well established.
- The solvent is not soluble in the conducting portion of the lung (anatomical dead space).

- The solvent is not metabolized to a great extent, and it is mainly eliminated via exhalation.
- Sample collection is conducted in a location unaffected by ambient levels of the solvent(s) of concern that may interfere and bias the measurements.
- The concentration in exhaled air is compatible with the analytical method allowing adequate sensitivity.
- Appropriate timing is strictly followed.

### *15.2.1.7 Examples of breath monitoring*

Two solvents are used to illustrate the use of breath monitoring in assessing solvent exposure: A solvent with minimal metabolism, tetrachloroethylene (PERC) and a solvent with significant metabolism, styrene. Both solvents are used extensively in industry with a great number of workers potentially exposed. As shown in Table 15.2.1.2, styrene is only minimally excreted through the lungs, whereas exhaled air is the major elimination route for PERC. Accordingly, end-exhaled air is recommended as a BEI for PERC, but not for styrene. Nevertheless, end-exhaled and mixed-exhaled air techniques have been studied in experimental settings and in real-life occupational environments for both solvents. A compilation of such studies is presented below.

**Table 15.2.1.2. Characteristics of styrene and tetrachloroethylene (PERC). [Information compiled from ACGIH, Documentation of Threshold Limit Values and Biological Limit Values, 1991[7]]**

| | STYRENE | PERC |
|---|---|---|
| Eliminated via lungs | ~5% | ~80-100% |
| Eliminated in the urine | ~90% | ~2% |
| Urinary metabolite(s) | Mandelic acid (MA),<br>Phenylglyoxylic acid (PA) | Trichloroacetic acid (TCA) |
| Blood/air partition coefficient (λ) | 32-55 | 9-15 |
| Pulmonary elimination half-lives ($T_{1/2}$) | 13-52 min<br>4-20 hr<br>3 days | 15 min<br>4 hrs<br>4 days |
| TLV (1998) | 50 ppm | 25 ppm |
| STEL (1998) | 100 ppm | 100 ppm |
| BEI (1998) | | |
| End-exhaled air | Not recommended | 10 ppm end-of-shift, after at least 2 shifts |
| Blood | 0.55 mg/L end-of-shift<br>0.02 mg/L prior-to-next-shift | 1 mg/L end-of-shift, after at least 2 shifts |
| Urine | MA=800 mg/g creatinine, end-of-shift<br>MA=300 mg/g creatinine, prior-to-next-shift<br>PA=240 mg/g creatinine, end-of-shift<br>PA=100 mg/g creatinine, prior-to-next-shift | TCA=7 mg/L |
| Major Industry | Reinforced Plastics | Dry Cleaning |

#### 15.2.1.7.1 PERC

Tetrachloroethylene (PERC) is used extensively in the textile industry as a dry cleaning aid and in metal processing as a metal degreaser. The major concern is on occupational exposures, but elevated indoor air concentrations of PERC have also been documented.[49] As a result, the potentially exposed population is very large, with a wide range of exposures. The uptake of PERC by the lung is high[51,52] because of its high solubility in blood and adipose. Most of it is eliminated via the lung, with very small amounts metabolized to trichloroacetic acid (TCA) and eliminated in the urine.[51,52] Even smaller amounts of trichloroethanol in the urine have been reported.[52]

A number of field studies have been published with similar designs, in which breathing zone air, blood, breath and urine were measured in groups of workers. In addition, chamber studies allowed measurements under predetermined consistent conditions. The emerging patterns formed the basis for recommendations for biologic monitoring.

In a study of thirty two workers,[9] end-exhaled air was measured at the beginning of the first shift of the week, after 15-30 min after the end of the shift on the 3rd and 5th day of the same week, and at the beginning of the first shift of the subsequent week. The authors required the subjects to hold their breaths for 5 seconds and collected only the last part of the breath to obtain alveolar air. These measurements correlated with personal breathing zone samples collected each day. The highest correlation was found between PERC and TCA in blood at the end of the workweek and the average exposure for the entire week ($R^2$=0.953). Among the non-invasive techniques, PERC in exhaled air collected 15-30 min after the end of the shift at the end of the week had an $R^2$ of 0.931, and was followed by TCA in urine ($R^2$=0.909).

In a field study of workers employed in dry cleaning,[53] twenty-four female and two male subjects were monitored. End-exhaled air and blood samples were collected at the beginning and at 30 minutes after the end of the 3rd workday of the week. On that same day, personal, shift-long, breathing zone samples were also collected from each worker. Urine samples were collected before and after the shift on a daily basis and analyzed for TCA. With a mean exposure of 20.8 ppm, no TCA was detected in any of the urine samples (no detection limit was reported). Among the three exposure parameters (breathing zone air, blood and end-exhaled air) only the correlation between the PERC concentration in the blood and in the breath was reported as statistically significant by the authors ($R^2$=0.77). The authors concluded that if the concentration of PERC in blood, 16 hours after the end of exposure, was below 1000 μg/L, the average exposure was likely to have been below 50 ppm.

A group of thirteen male dry cleaners[40] was followed for a week with daily shift-long personal (badge) monitoring; mixed-exhaled air collected daily before- and after-shift; and mixed-exhaled air, blood and urine measurements on the morning after the last shift. Results indicated strong correlations between PERC concentrations in mixed-exhaled air and blood ($R^2$=0.944), as well as mixed-exhaled air and personal air samples collected either after the shift ($R^2$=0.885), or on the following morning ($R^2$=0.770). Mixed-exhaled air measurements collected either at the end of the last shift of the week ($R^2$=0.814) or in the morning following the last shift ($R^2$=0.764) were better indicators of exposure over the entire week than either blood ($R^2$=0.697) or urine ($R^2$=0.678). The authors recommended consideration of mixed-exhaled air as a potential BEI.

In addition to field studies, controlled human exposures to PERC have been reported. In this type of studies, subjects were exposed to various constant levels of PERC for different lengths of time with or without exercise. Samples of blood, exhaled air and urine were collected according to a variety of schedules both during exposure and for varying lengths

of time after exposure. One study involved single exposures, whereas in others at least some of the subjects participated more than once.

In one of the first chamber studies, nine male volunteers were exposed to 100-200 ppm of PERC for 80 to 180 minutes. Concentrations of PERC in exhaled air were measured over time.[54] The authors demonstrated that exposures of similar duration resulted in similar decay curves of PERC concentrations in breath after exposure. In addition, the data showed that the length of time that PERC was measurable in exhaled air was proportional to both the concentration and the duration of the exposure.

The same group[55] exposed sixteen males to single and repeated concentrations of 100 ppm PERC for periods of 7 hrs/day over a 5-day workweek. Alveolar air samples were collected over a period of up to 300 hours post-exposure. The authors observed higher alveolar air concentration in subjects with greater body mass. They recommended measurement of PERC in alveolar air 2-16 hours post-exposure to assess the average exposure.

Six healthy males were exposed to 72 and 144 ppm, as well as 142 ppm with 2 half hours of 100 W of exercise.[51] Uptake of PERC was calculated from the minute volume and the concentration of PERC in breath. PERC was measured in breath and blood and TCA was measured in blood and urine. During the first hour of exposure the uptake was higher by 25% than during the last hour and retention decreased with time and with exercise. The mean ratio of the concentration of PERC in venous blood and the concentration of PERC in mixed exhaled air was 23. Assuming a mix-exhaled air to end-exhaled air ratio of 0.71, they estimated a blood/air partition coefficient of 16. After 20 hours from the end of exposure the half-life was estimated to be 12-16 hours, after 50 hours the half-life was estimated to be 30-40 hours and after 100 hours it was estimated at approximately 55 hours. The authors estimated that 80-100% of PERC was excreted unchanged through the lung, and about 2% was excreted as TCA in the urine.

Simple and multiple linear regression analyses were applied to the data collected above to predict the best estimator of exposure to PERC.[56,57] PERC in blood was the best predictor followed by PERC in exhaled air. TCA per gram of creatinine in urine was the least reliable predictor.

Twenty four subjects were exposed to 100, 150 and 200 ppm of PERC for 1 to 8 hours.[52] The concentration of PERC in alveolar air decreased rapidly during the first hours of the post-exposure period, but 2 weeks were required for complete elimination following an exposure of 100 ppm for 8 hours. The authors were the first to demonstrate that the rate of rise and of decay of the alveolar concentration with respect to time was independent of the level of the inspired concentration during exposure.

The experimental data from the above study were used in a pharmacokinetic model[58] that divided the body into four tissue compartments: the Vessel Rich Group (VRG), the Muscle Group (MG), the Fat Group (FG) and the Vessel Poor Group (VPG). No metabolic pathway was considered. After 20 hours from the end of the exposure the concentration of PERC in alveolar air was related to release from the FG with an approximate half-life of 71.5 hours. The model predicted small differences in alveolar concentrations due to differences in body weight, height and fat tissue. The authors provided a nomogram to predict mean exposure given the exposure duration (0-8 hrs), time since end of exposure (0-8 hrs) and post-exposure measurement of the alveolar air concentration.

A perfusion-limited, four compartment model was used to simulate the effect of several confounding factors on the levels of several contaminants in the breath, including PERC.[6] The model, which considered metabolic clearance, consisted of a pulmonary compartment, a vessel-rich group, a group of low-perfused tissues containing muscles and skin, and a group of poorly perfused fatty tissues. The authors predicted that intra-day fluctuations in exposure would have a large effect on breath sampled just after the shift, but that the

effect would be negligible the next morning. Breath samples collected the next morning would reflect exposure on preceding days and the measurements would be independent of level of physical exertion. Finally, individual differences in body build and metabolism would have the greatest effect on breath levels, with the largest variations expected for metabolized solvents.

Experimental data were used in a model,[59] which divided the body into four tissue compartments: vessel rich group, muscle group, fat group and liver. Metabolism was assumed to take place in the liver as a combination of a linear metabolic component and a Michaelis-Menten component. Metabolic parameters and partition coefficients determined for rats were scaled for body weight and were used in fitting results for humans. The model fit very well the data reported from other controlled human exposure studies.[51,52,54]

The BEI committee of the ACGIH recommends[2] three indices of PERC exposure:

1) PERC in end-exhaled air collected prior to the shift after at least 2 consecutive workdays.

2) PERC in blood collected prior to the shift after at least 2 consecutive workdays.

3) Trichloroacetic acid (TCA) in urine voided at the end of the workweek. This index is recommended only as a screening test because of the variability associated with urinary excretion of TCA and, as such, no creatinine correction is necessary.

15.2.1.7.2 Styrene

The highest exposures to styrene take place in plants manufacturing glass-reinforced plastics, particularly during lamination, where there is considerable evaporation of styrene. Most of the inhaled styrene is absorbed and retained in the body. The first step in the major metabolic pathway is the formation of styrene-7,8-oxide (phenyloxirane), a reaction catalyzed by Cytochrome P-450 in the liver. The oxide is then hydrated to styrene glycol (1-phenyl-1,2-ethanediol) by microsomal epoxide hydratase. The styrene glycol is conjugated with beta-glucuronic acid, or is oxidized to mandelic acid and further to phenylglyoxylic acid. In humans, less than 5% of absorbed styrene is eliminated via the lung, whereas 90% is eliminated in the urine as mandelic and phenylglyoxylic acids.

A number of chamber and field studies have been reported, where blood, exhaled air and urine have been studied as possible biological indicators. Although conditions and study design differed among studies, certain consistent patterns emerged, which formed the basis for subsequent recommendations.

In an early series of chamber exposures,[60] nine healthy males were exposed to styrene concentrations of 50 to 370 ppm for periods ranging from 1 to 7 hours. Alveolar air samples were collected during and for 8 hours following exposure. The authors showed that the amounts of styrene present in the breath were directly related to the level and the duration of exposure.

In another study,[61] fourteen healthy males were exposed to 50 and 150 ppm of styrene in inspired air during rest and light (50 W) physical exercise. The duration of exposure for each styrene concentration was 30 minutes, with total pulmonary ventilation, cardiac output, and styrene concentration in alveolar air, arterial blood and venous blood measured during and after exposure. During exposure at rest to either 50 or 150 ppm, the alveolar air concentrations were about 20% of the inspired air concentrations. When alveolar ventilation almost tripled with exercise at 50 W, the alveolar air concentrations increased slightly, whereas arterial blood concentrations almost tripled. Alveolar air concentrations reached a plateau within a minute or so, that persisted throughout the 30-minute period. Arterial blood concentrations, however, rose rapidly during each such period and even more with increasing exercise intensity (50, 100 and 150 W). Venous blood concentrations also rose sharply during the 30-minute exposure periods without ever reaching a plateau. No linear relationship was found between exposure duration and arterial and venous blood concentrations,

even after log-log transformations. The decay of styrene concentrations in alveolar air and in venous and arterial blood was slower in the first 30 to 50 minutes than later on. The authors concluded that post-exposure alveolar air measurements were poor predictors of exposures to styrene, whereas arterial blood would be the best index of exposure. For practical reasons, they recommended capillary blood samples from fingertips as a surrogate for arterial blood.

In a follow-up study[62] using the same experimental set up as above, seven male subjects were exposed to 50 ppm (210 mg/m$^3$) of styrene in inspired air for 30 minutes at rest, followed by 30 minute exposure periods under exertion at intensities of 50, 100 and 150 W. The mean alveolar air concentration at the end of the first 30-minute period was 16% of the concentration in the inspired air and 23% at the end of the final period. Both the arterial and venous blood concentrations of styrene rose with increasing workload during exposure with no equilibrium achieved between alveolar air and arterial blood during 2 hours of total exposure. Weak correlations were found for the amount of styrene taken up per kg of body weight and the concentrations of styrene in alveolar air 0.5 and 2 hours after exposure. The elimination was faster during periods of exercise than during rest periods, leading to the authors' recommendation for leisure time physical activity for people exposed to solvents as a means to enhance elimination.

In another study,[63] four male volunteers were exposed to 80 ppm of styrene in a chamber for 6 hours. Venous blood samples were collected during exposure and a nearly simultaneous set of 10 blood and mixed exhaled air samples were collected following exposure for up to 40 hours. Blood levels during exposure rose rapidly and reached an almost constant level by the end of the 6 hours. Following exposure, the results showed that styrene was cleared from the blood according to a linear two-compartment pharmacokinetic model, with half-life values of 0.58 and 13 hours, for the rapid and slow elimination phases, respectively.

Retention of styrene was studied in a chamber setting where healthy males were exposed to either constant or fluctuating air concentrations of styrene.[26] A computer-controlled system was used to generate time-varying air concentrations over 4-5 hrs with a mean air concentration of 50 ppm. End-exhaled air measurements taken throughout the exposure period showed styrene retention of 93.5% during constant exposures, but higher retention (96-97%) during fluctuating exposures. The authors speculated that the difference in retention was related to non-steady-state behavior of styrene in the richly perfused tissues.

In summary, pulmonary retention during exposure is about 60-70%[62,64] with exhaled concentrations accounting for 25-35%.[60] Higher retention (96-97%) was observed when volunteers were exposed to fluctuating, rather than constant, air concentrations.[26] Following exposure, the desaturation curve shows two exponential decays with half-lives of 13 minutes[60] to 52 minutes[63] for the rapid elimination phase and 4 hours[60] to 20 hours[63] for the slower elimination phase. A third compartment can be defined to represent elimination from the adipose tissue with a half-life of 3 days.[62]

Measurements of styrene in venous blood at the end of the workshift and/or prior to the next shift have been recommended by the ACGIH.[2] Monitoring of urinary metabolites of styrene has also been recommended by the ACGIH[2] at the end of the week, even though they are not specific to styrene exposure. Styrene in exhaled air after the end of exposure was not recommended as a BEI because the levels would be too low to detect, leading to uncertainties.

## Terminology

| | |
|---|---|
| ACGIH | American Conference of Governmental Industrial Hygienists |
| Alveolar air | Gas in the alveoli |

| | |
|---|---|
| Alveolar ventilation | Inhaled air available for gas exchange in 1 min |
| Anatomical dead space | Volume (~150 mL) of conducting airways where no gas exchange occurs |
| BEI | Biological Exposure Index. Reference value developed by ACGIH |
| Blood/air partition coefficient | Ratio of the vapor concentration in blood and in air at 37ºC |
| Breathing frequency | 15 breaths per minute at rest, increases with physical exertion |
| Conductive zone of lungs | First segment of the airways, which includes the trachea, bronchi, terminal bronchioles |
| End-exhaled air | Operationally defined as the last portion of an exhalation to simulate alveolar air |
| Exhaled air volume | Approx. 500 mL at rest, increases with workload |
| GC-MS | Gas Chromatography Mass Spectrometry |
| Minute Volume Ventilation | The volume of air exhaled in 1 min, approx. 7,500 mL |
| Mixed-exhaled air | Entire volume of exhaled air collected from alveolar space and anatomically dead space |
| Respiratory zone | Consists of respiratory bronchioles and alveolar ducts where gas exchange takes place. Approx. 2,500 mL |
| STEL | Short-Term Exposure Limit. The concentration to which most workers may be exposed continuously for a short period of time without adverse effects. Issued by ACGIH |
| Tidal Volume | Volume of exhaled air; ~500 mL at rest |
| TLV | Threshold Limit Value. Airborne concentration of a substance to which most workers may be exposed without adverse health effects. Established by ACGIH and intended as guideline. |

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## 15.2.2 A SIMPLE TEST TO DETERMINE TOXICITY USING BACTERIA

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### 15.2.2.1 Introduction

The author has developed a simple, inexpensive and rapid method to measure toxicity using a bacterial indicator. This paper describes this test in detail. It also reviews the field of alternative tests for toxicity, tests not involving viable animals.

### 15.2.2.2 Toxicity defined

In toxicology, a compound is defined to be toxic if it damages living organisms. There is no chemical definition of toxicity. Classically, toxicity is determined from the LD50 for animals, the amount of the compound that kills 50% of the test animals (Rodericks, 1992). Typically varying concentrations of the toxin are given to groups of 10 animals and a single test can take 80 to 100 animals. Tests can take several weeks. Animal tests are expensive. The animals must be cared for and trained personnel are required. The animals are force fed the toxin so it passes through the acidic stomach or the toxins are introduced by intraperitoneal injection. This can alter the activity of the toxin. The results from animal tests are difficult to interpret since animals can die of causes other than the toxic chemical and analysis of the data usually requires sophisticated statistical analysis. In the United States it is estimated that 30 million animals die each year testing for toxic chemicals. Many question the value of animal tests (Ruelius, 1987). Results vary dramatically between strains of animals. Results with closely related species, rats and mice, often don’t agree well. The drug Thalidomide many years ago was tested in animals and was found to be harmless. The drug had tragic effects on humans. And, of course, animal tests do not note if the animal becomes ill from the toxin. They only note if the toxin kills the animal.

Often it is not known why the chemical is toxic. It is simply observed that animals coming in contact with the toxin die at abnormally high rates. Obviously the first step in a study of a toxic chemical, is simply to determine if it is toxic.

Most animal tests are run with rats and mice, the usual laboratory animals. Rats and mice are rodents and they have a caecum, a chamber that opens off the small intestine. The caecum contains many microorganisms. It is not known what the role of the aecum could be in handling toxic chemicals. This may influence results with rats and mice.

Alternatives to animal tests are sought. The sand flea *Daphnia magna* is used extensively. Tests are inexpensive, most the animal rights advocates are not offended. *Daphnia* tests are difficult to perform and require highly skilled personnel. The tests typically take two days (Stephenson, 1990). Once it has been determined that the toxin kills the juvenile cells, it can be determined if the toxin interferes with maturation of the juveniles, with reproduction in the adults, or if the toxin simply inhibits growth. However, nearly all reports simply deal with death of the animals. *Ceriodaphnia dubia* offers another approach to this sort of test (Jung and Bitton,1997). There is a version of this test called Ceriofast™ that takes only one hour. *Daphnia* testing requires laboratory skills that typically lab workers do not develop. The juvenile insects must be isolated using a dissecting microscope. The animals must be monitored to determine if they are living. It is necessary to watch the culture to know when to isolate the juveniles.

In Europe many tests with animal and human cells have been developed (Clemendson et al., 1996). These tests are effective, provide values comparable to those obtained with animals. But they require that the animal cells be grown in culture. This requires a sophisticated laboratory and well trained personnel. Again the tests take several days. Some tests take up to a week. Damage to the cells is determined in a variety of ways. The ability of cells to reduce the thiazole tetrazolium dye, MTT, is noted; the activity of an enzyme, often lactic dehydrogenase, is determined; the ability of the cell to take up some dyes or to retain other dyes is observed. Often morphological changes are noted to determine if the cells are damaged. These techniques can be quite complicated and require well trained personnel and a sophisticated, well equipped, laboratory.

There are several reports using animal cells to test the MEIC chemicals (Shrivastava et al., 1992; Rommert et al., 1994; Rouget et al., 1993). Shrivastava et al., used freshly isolated rat hepatocyte cells and two transformed cell lines. Comparable results were obtained with the three cell types indicating that rat hepatocytes are no more resistant to toxic chemicals than are other cells. They determined viability of the cells with 1) morphological studies; 2) lactic dehydrogenase activity in the hepatocytes; 3) the ability of the cells to take up the dye tryptan blue. They got much the same results with the three techniques. Rommert et al., simply determined the effect of the toxins on the ability of the cells to replicate. They determined cell numbers with a Coulter Counter after 48 hours. Rouget et al., followed the effect of the toxins by determining the ability of cells to reduce the tetrazolium dye MTT and the ability of cells to take up the dye neutral red. The authors maintained that the two methods provided comparable results.

In Europe tests with rotifers, cysts from aquatic invertebrates (Calleja et al., 1994), development of onion roots have been tried but none have been accepted (Persoone et al., 1994; Snell and Persoone, 1989; Fresjog, 1985; Gaggi et al. 1995). Jaffe (1995) has proposed a method using the survival of a protozoan. Toxic chemicals kill the protozoa. The test requires a Coulter particle counter to determine the numbers of viable protozoa.

Tests using bacteria as the indicator organism are on the market. Microtox™ uses a bioluminescent marine bacterium (Bullich et al., 1990). The bacteria, when growing in high numbers, emit light. Toxic chemicals inhibit the production of light. The test requires a luminometer to measure light production and a refrigerated water bath to grow the cells at 15°C. The results are difficult to interpret. The ability of the cells to emit light gradually decreases and a control to follow production of light in the absence of a toxin must be run. And the value for this enters into the calculations. Typically the calculations require at least a half hour to carry out (Ribo and Kaiser, 1987). A sophisticated computer program is used to analyze the results. The initial cost of the kit is significant, but it is inexpensive to use once this cost is absorbed (Elanabarwy et al., 1988). The test appears to be challenge for an MS level graduate student at our university. It is not certain why toxic chemicals inhibit light production but it is assumed that the chemicals damage the cytoplasmic membrane and reduce electron transport to the pigments responsible for light production. This method is very popular as a “first test”, to see if a chemical need be investigated further. The test has been used to determine the toxicity of more than a thousand chemicals (Kaiser and Palabrica, 1991). There is a European version of the same text, Biotox™ (Kahru and Borchardt, 1994). Thomulka et al. (1995) have proposed a variation on this test.

Polytox™ uses a consortium of bacteria from sewage sludge. Toxic chemicals inhibit the oxidation of a carbon source (presumably glucose). Presumably the toxic chemicals damage the cellular membrane and the associated cytochromes involved in electron transport. Oxidation of the substrate is followed with a respirometer or with oxygen electrodes. Both these methods are complicated and expensive to set up and to maintain. Again, at our university, this technique appears to be appropriate for MS level graduate students. This test

is used to determine if chemicals will harm sewage treatment plants. It is rarely used to determine the toxicity of compounds. The test provides values comparable to those observed using sewage sludge as the source of bacteria (Sun et al., 1993; Toussaint et al., 1995; Elnabarawy et al., 1988).

Toxitrak™ has been used to determine toxicity in water samples. There is a report (Gupta and Karuppiah, 1995) in which they tested water samples from a river in Maryland using this method, Microtox™, *Ceriodaphnia dubia* (a variation of the standard *Daphnia* test), and this test. With four samples, they got comparable results. The Toxitrak test is not used extensively. Apparently the test follows the reduction of dye resazurin by cells of *Bacillus cereus* (Liu, 1989).

Dozens of tests using bacteria as indicator organisms in toxicity studies have been proposed (Bitton and Dutka, 1986). Very few of these have been characterized extensively. Bacterial tests are inexpensive, inoffensive to animal rights groups, the cells are easy to grow and to prepare. And it is thought there is enough universality at the biochemical level, that a compound that is toxic for a bacterium will be toxic for a higher organism.

Ideally an alternative test for toxic chemicals would be inexpensive. It would not require any specialized equipment, only equipment normally found in a laboratory should be required. The test should not require that the personnel be specially trained in the techniques. It should involve only the procedures that any laboratory worker would know. It should be the sort of test that any laboratory with a tangential interest in toxicology could carry out. Water quality laboratories, industrial safety laboratories, agricultural research laboratories, should be able to carry out the procedure. Small samples should be tested. Tests with fish often involve quite large volumes of toxic chemicals and this can limit their utility. This has been discussed in detail by Blaise (1991).

The sensitivity of a test can be a problem. Every test for toxic chemicals has some chemicals that the test is very sensitive to. And every test has some toxic chemicals that it is not sensitive to. Often several tests will be run and the median value for toxicity is judged to be representative for the toxicity of that chemical. It can be argued that if a test system is extremely sensitive to a chemical, than it must be concluded that this chemical could be toxic for humans at this very low level. If it damages one life form, it could very well damage another. Tests with live animals, LD50 tests, are usually not as sensitive as tests with *Daphnia*, with animal cells, with bacterial tests. Tests with live animals usually involve injecting the toxin into the stomach and it is not always certain what effect the acidic conditions of the stomach may have on the toxin. And humans rarely come in contact with toxins in this fashion. It can be argued that this sort of animal test is not indicative of the toxicity of the chemical (Ruelius, 1987). On the other hand, chemical analysis is often much more sensitive than any toxicity test. There are reports of chemicals in the range of parts per trillion yet few tests for toxic chemicals can detect less than parts per million or occasionally, parts per billion. Yet we see public policy formulated on the basis of these chemical tests.

### 15.2.2.3 An alternative

A simple, inexpensive and rapid test to determine the toxicity of chemicals has been developed. (Botsford, 1997, 1998, 1999). The assay uses the bacterium *Rhizobium meliloti* as the indicator organism. This bacterium reduces the thiazole tetrazolium dye, MTT (3-[4,5-dimethylthiazole-2-y]-2,5-biphenyl tetrazolium bromide) readily. The dye is almost completely reduced after a 20 minute incubation at 30ºC. The dye turns dark blue when reduced and this can be followed readily with a simple spectrophotometer. Reduction of the dye is inhibited by toxic compounds. More than 200 compounds have been tested and the results obtained with this test are comparable to those obtained with other tests (Botsford, 2000a).

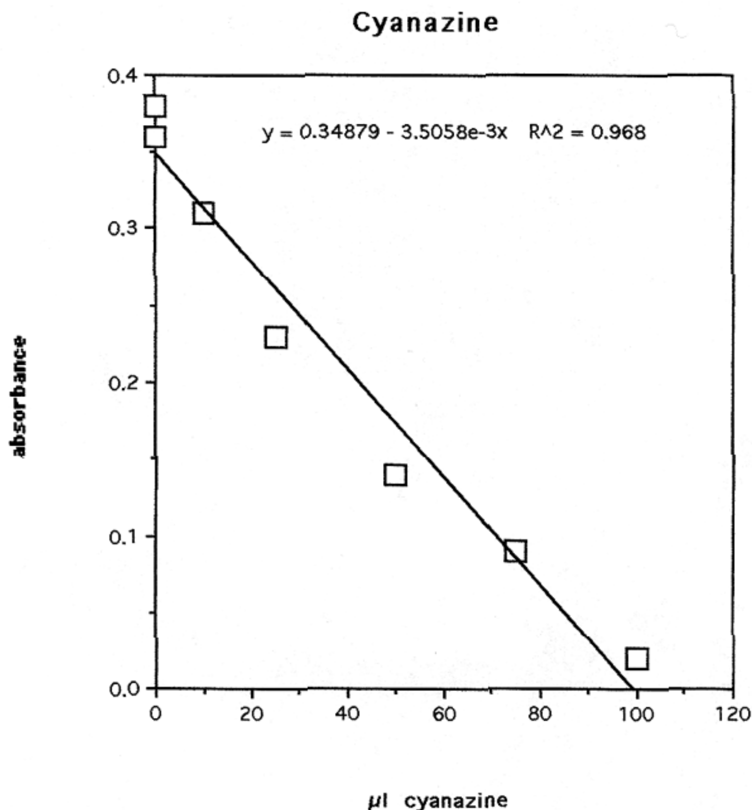


Figure 15.2.2.1. Cyanazine is in units of µl. Cyanazine is a herbicide. The toxicity of cyanazine is: IC50 = [Y/2 -B]/m; Y = 0.35, 0.37 = 0.36, 0.36/2 = 0.18; B = 0.3487 m = 3.5058x$10^{-3}$; IC50 = 48.1; 48.1 µl = ([48.1 x $10^{-6}$ l]/test tube) x([3300 mg]/l) x [1 test tube]/3.3 ml)= 48.1 µg/ml = 48.1 mg/l = 48.1 ppm.

Since alternative tests don't involve killing test animals, results are reported as IC50 values (inhibitory concentration, 50%), the concentration that results in death of 50% of the cells, in 50% inhibition of oxygen consumption, 50% inhibition of reduction of the dye in the *Rhizobium* assay.

Cells are grown overnight in a simple defined medium with 0.1% casamino acids. Cells are collected by centrifugation in a refrigerated preparative centrifuge. Cells are washed once with 0.01 M (pH 7.5) potassium phosphate buffer and are then diluted in this same buffer. Volumes of the toxin (usually 5 to 1000 µl) are put into test tubes. The volume of the toxin is made up to 2.1 ml with water. 0.1 ml of 0.1 M Tris or Bicene buffer, pH 7.5, is added. The absorbancy at 550 nm is determined. 0.1 ml MTT (the tetrazolium dye) is added (3.2 mM). The tubes are incubated 20 minutes at 30°C. The absorbance is read a second time. The difference in the absorbance at time = 0 and at time = 20 min is determined. The results are plotted, the difference in absorbance of each tube with a different volume of the toxic chemical on the X axis versus the absorbance on the Y axis (Figure 15.2.2.1). A regression line is fit to the plot and if the regression coefficient is less than 0.8, the experiment is rejected. From the average value of the controls (tubes with no toxin), the Y intercept forms the regression equation and the slope, the volume of toxin inhibits reduction of the dye 50% can be readily calculated. For some toxins, better results are obtained if the log of

the volume of the toxin is plotted. From this the log of the volume corresponding to 50% inhibition of reduction can be determined and the antilog of this value provides the volume. In this laboratory the data is plotted both linearly and logarithmically and the best method providing the regression coefficient closest to 1.00 is chosen. From the concentration of the toxic compound, the volume found to inhibit reduction of the dye could be converted to mg/ml present in the test tube. From this the inhibitory concentration causing 50% inhibition, the IC50, can be calculated. The test has been described in detail (Botsford et al., 1997; Botsford 1998; Botsford, 1999).

A method to lyophilize cells has been developed and cells are active for at least a month after being lyophilized (Robertson, 1996).

This test for toxic chemicals is very inexpensive. It can be carried out by unskilled personnel. It is rapid, taking less than an hour to determine the toxicity of a compound. It provides values comparable to more conventional tests (Botsford, 2000a). It provides a sort of toxicological triage, a method to rapidly determine if something is toxic and should be investigated further. Since it uses bacteria rather than animals as an indicator, it is acceptable with animal rights advocates. The test has been patented and the author is seeking a firm to market the test. In the meantime, it can be carried out by any laboratory equipped to grow bacteria.

Since this test uses a bacterium as the indicator organism. It can be argued that the results with a bacterium are not applicable to humans. But tests with rats and mice may not be applicable to humans (Ruelius, 1987). For example, in a test of 10 chemicals comparing results with mice, rats and five cellular assays, the toxicity of three of compounds differed by at least an order magnitude when rats and mice were compared (Ekwall et al., 1989). Such anomalies been observed with many drugs, that drugs were up to 12× more toxic for humans than for rats and mice (Brodie and Reid, 1967). At a biochemical level, a compound that damages the cytoplasmic membrane of a bacterium will also damage the cytoplasmic membrane of a human or any other organism. This test has been compared with 20 other tests for toxic chemicals and comparable results (similar IC50 values) are obtained (Botsford, 2000a). No one test finds all chemicals to be toxic. Every assay for toxic chemicals is blind to some compounds and conversely every assay for toxic chemicals is much more sensitive to some compounds than are other tests. Thus toxicologists desire "batteries of tests", a compound is not considered to be toxic until it has proven to be toxic in 3 or 4 systems.

#### 15.2.2.4 Chemicals tested

In Table 15.2.2.1, the values for the more than 200 chemicals tested in this laboratory are reported. This includes a series of organic chemicals, chemicals that are reported in the literature as being toxic. It was noted that aromatics, phenol, benzene and toluene are not very toxic. But when they have substituents, a phenoxy group, chlorine, methyl or nitro they become quite toxic. Solvents were tested for toxicity and most solvents are not very toxic. We have found we can dissolve toxic compounds in solvents (methanol and DMSO) and find no change in the toxicity. Apparently the toxin is so much more toxic than the solvent that only the toxin affects the mechanism affording the assay. Many drugs were also tested and it is significant that many of these drugs are toxic for the bacterium and presumably would be toxic for patients receiving them. Often it is noted that when a drug is taken, an individual simply does not feel well. This could be due to toxicity of the drug. It was found that many alkaloids are toxic and then it was shown that many herbs are also toxic. Presumably the herbs contain toxic levels of some alkaloids. Some of these herbs are toxic enough that if mega herb therapy ever becomes popular, there could be problems from the toxicity of the herbs. It was found that divalent cations are toxic for the system. It was found that small amounts of EDTA could relieve this toxicity in soil and water samples. This will be dis-

cussed. Neither lead nor arsenic acid were found to be toxic. Finally 30 herbicides were tested for toxicity (Hillaker, 1996). The toxicity determined by this method was compared with the toxicity reported by the manufacturers using rats, ducks, and quail in classical LD50 tests; and with trout fingerlings and with *Daphnia*. The tests with trout fingerlings and with *Daphnia* were much more sensitive than the tests with animals. The tests with *Rhizobium* were less sensitive than with *Daphnia* and the trout fingerlings for most of the herbicides but were always more sensitive than tests with animals.

**Table 15.2.2.1. Toxicity of chemicals tested**

| | n | Ave. | Var. | | n | Ave. | Var. |
|---|---|---|---|---|---|---|---|
| 1,1,1-trichloroethane | 20 | 229 | 10 | pentachlorophenol | 13 | 0.143 | 50 |
| 1,1,1-tricholorethylene | 13 | 66.3 | 27 | 2-methyl resourcinol | 4 | 404 | 19 |
| 1,2-dichloroethane | 2 | 146.5 | | m-cresol (mono methyl) | 6 | 98.9 | 14 |
| 1,10-phenanthroline | 5 | 42.7 | | o-cresol | 22 | 190 | 29 |
| acetic acid | 8 | 429 | 10 | p-cresol | 4 | 386 | 15 |
| acetonitrile | 3 | >3300 | | 2,4-dinitro-o-cresol | 7 | 12.8 | 39 |
| butyl amine | 2 | 271 | | 2,6-dinitro-p-cresol | 7 | 33.5 | 35 |
| coumarin | 7 | 222 | 21 | 4,6-dinitrocresol | 5 | 6.83 | 31 |
| CTAB (detergent) | 7 | 0.90 | 36 | toluene | 4 | 217 | 8 |
| cyclohexyl amine | 9 | 234 | 5 | toluidine | 6 | 1493 | 26 |
| diethyl amine | 2 | 194 | | trihydroxytoluene | 8 | 52.4 | 34 |
| dimethyl amino ethanol | 2 | >1200 | | dinitrotoluene | 4 | 66.7 | 6 |
| dibromethane | 8 | 217 | 7 | trinitrotoluene | 8 | 52.4 | 34 |
| EDTA | 6 | 3788 | 14 | benzene | 4 | 799 | 18 |
| ethyl acetate | 11 | 1107 | 17 | benzyl chloride | 8 | 60.6 | 8 |
| ethyl benzene | 8 | 71 | 6 | chlorobenzene | 12 | 23.9 | 26 |
| ethanol amine | 8 | 251 | 16 | 1,2,4-trimethyl benzene | 14 | 37.6 | 40 |
| hexachlorophene | 7 | 0.077 | 9 | dimethyl amino benzaldehyde | 1 | 236 | |
| hydrogen peroxide | 10 | 164 | 28 | trichlorobenzene | 5 | 14.7 | 18 |
| hydroxyl amine | 13 | 566 | 20 | p-amino benzaldedhyde | 3 | 156 | 4 |
| ibenofuran | 4 | 9.61 | 20 | 1,2-dichlorobenzene | 11 | 37.7 | 5 |
| indole | 3 | 501 | 5 | 1,3-dichlorobenzene | 7 | 41.4 | 11 |
| isonicotinic acid | 5 | 287 | 18 | 1,4-dinitrobenzene | 3 | 313 | 8 |
| octane | 5 | 161 | 20 | pseudocumine | 14 | 37.6 | 40 |
| α-naphthol | 9 | 56.9 | 34 | p-dimethyl amino benzaldehyde | 7 | 177 | 5 |
| potassium ferricyanide | 5 | 3600 | 15 | p-amino benzoic acid | 3 | 291 | 10 |
| pentanol | 4 | 1627 | 22 | p-OH benzoic acid | 5 | 252 | 27 |

| | n | Ave. | Var. | | n | Ave. | Var. |
|---|---|---|---|---|---|---|---|
| phenanthroline | 5 | 43.3 | 20 | 2-OH benzoic acid (salicylic acid) | 3 | 217 | 13 |
| Tris (Trizma, pH 7.5) | 3 | 5447 | 16 | p-amino benzoic acid | 4 | 307 | 2 |
| MOPS 3-[N-morpholino] propane sulfonate | 5 | 13090 | 27 | chlorobenzoic acid | 6 | 233 | 19 |
| tetrachloroethylene | 16 | 91.2 | 21 | 4-chlorobenzoic acid | 5 | 234 | 14 |
| tetrachloromethane | 10 | 1791 | 12 | 3-phenoxybenzoic acid | 4 | 62.7 | 25 |
| salicylic acid | 4 | 198 | 20 | 3(3,5-dichlorophenoxy) benzoic acid | 5 | 41.9 | 20 |
| sulfosalicylic acid | 11 | 1107 | 17 | 3(4-chlorophenoxy) benzoic acid | 7 | 32.9 | 43 |
| xylene | 4 | 132 | 27 | 3(4-methyl phenoxy) benzoic acid | 6 | 92.1 | 9 |
| phenol | 16 | 1223 | 28 | 3(3,4-dichlorophenoxy) benzoic acid | 4 | 45.9 | 24 |
| 4-chlorophenol | 6 | 186 | 23 | **Solvents** | | | |
| 3-chlorophenol | 6 | 3.21 | 45 | acetone | 18 | 123000 | 24 |
| 3-methyl-4-nitro phenol | 4 | 54.9 | 27 | acetic acid | 8 | 429 | 10 |
| 2,4-dichlorophenol | 4 | 68.7 | 5 | coumarin | 7 | 222 | 21 |
| 3,4-dichlorophenol | 5 | 82.3 | 74 | chloroform | 10 | 1550 | 22 |
| 3,5-dichlorophenol | 5 | 174 | 37 | ethanol | 20 | 75581 | 28 |
| 2,3-dimethyl phenol | 10 | 120 | 5 | DMSO | 13 | 87629 | 31 |
| 2,4-dimethyl phenol | 11 | 71.4 | 28 | dichloroethane | 28 | 237 | 38 |
| 2,4,6-trimethyl phenol | 10 | 123 | 9 | dibromomethane | 8 | 217 | 15 |
| 3,4,5-trimethyl phenol | 6 | 49.5 | 4 | isopropyl alcohol | 3 | 57000 | 19 |
| 2,3,6-trimethyl phenol | 6 | 151 | 9 | methanol | 4 | 68172 | 13 |
| dimethyl amino methyl phenol | 10 | 413 | 24 | pentanol | 4 | 1623 | 22 |
| trichlorophenol | 45 | 8.24 | 38 | ethylene glycol | 5 | 212700 | 12 |
| o-nitrophenol | 13 | 37.6 | 33 | | | | |
| **Herbs** | | | | **Detergents** | | | |
| Aloe Vera | 4 | 219 | 4 | CTAB | 7 | 0.90 | 36 |
| Cayenne | 4 | 700 | 1 | SDS | 8 | 31.4 | 14 |
| Echinacea | 7 | 451 | 1 | Tween 20 | 5 | 2896 | 22 |
| Echinacea root | 5 | 939 | 4 | **Insecticides** | | | |
| Elderberry | 4 | 291 | 1 | Lindane | 8 | 41.1 | 28 |
| Evening Primrose | 11 | 35 | 17 | Malathion | 14 | 37.1 | 40 |
| Myrrh | 6 | 71.2 | 3 | Diazanon | 18 | 35.6 | 24 |

| | n | Ave. | Var. |
|---|---|---|---|
| **Herbs continuation** | | | |
| Gensing Drink | 9 | 285 | 9 |
| Royal Jelly | 6 | 1641 | 22 |
| Saw Palmetto | 21 | 149 | 24 |
| Chromium Picolinate | 6 | 206 | 7 |
| St. John's Wort | 20 | 192 | 12 |
| St. John's Wort | 6 | 218 | 4 |
| St. John's Wort | 6 | 639 | 17 |
| Thyme | 4 | 338 | 5 |
| Valarian Root | 12 | 208 | 13 |
| Valarian Root | 5 | 200 | 4 |
| Car's Claw, Garlic Oil, Ginko Bioloba, Ginseng, Golden Seal, and Herb Garlic were found not to be toxic (less than 2000 ppm). Herbs listed more than once were obtained from different suppliers. | | | |
| **Herbicides** | | | |
| 2,4-D | 9 | 347 | 39 |
| Alachlor | 6 | 111 | 8 |
| Bensulide | 7 | 53.7 | 18 |
| Bromoxynil | 8 | 26.0 | 19 |
| Clomazone | 15 | 23.8 | 16 |
| Cyanizine | 13 | 781 | 27 |
| DCPA | 6 | 42719 | |
| Dicamba | 6 | >1200 | |
| Diuron | 9 | 87.0 | 13 |
| EPTC | 9 | 51.4 | 6 |
| Ethalfluralin | 6 | >1200 | |
| Fuazifop-P | 6 | >1200 | |
| Glyphosate | 6 | 18.1 | 12 |
| Imazapyr | 6 | 229 | 1 |
| Imazethapyr | 10 | 35314 | |
| Isoxaben | 16 | 365 | 15 |
| Mepiquat-Cl | 6 | >1200 | |
| Metasulfuron | 6 | >1200 | |

| | n | Ave. | Var. |
|---|---|---|---|
| **Drugs** | | | |
| acetomenaphen | 9 | 249 | 20 |
| acetylsalicylic acid | 8 | 447 | 11 |
| aspirin (Bayer) | 10 | 170 | 21 |
| Amoxicilin | | | |
| Cyprus | 3 | 4412 | 37 |
| Jordan | 5 | 4501 | 51 |
| Mexico | 9 | 4257 | 9 |
| America | 4 | 7393 | 52 |
| chloramphenicol | 5 | 1400 | 24 |
| gentamycin | 5 | 0.68 | 7 |
| GEA-1 (nitroso drug) | 5 | 50.5 | 39 |
| Sin-1 (nitroso drug) | 2 | >1200 | |
| ibuprofen | 6 | 120 | 8 |
| isoproterenol | 1 | 1770 | |
| kanamycin | 5 | 0.85 | 14 |
| naproxin | 3 | 296 | 41 |
| neomycin | 8 | 0.438 | 31 |
| novobiocin | 2 | 2063 | |
| Orudis | 6 | 114 | 14 |
| orphenadrine | 6 | 472 | 32 |
| paparavine | 7 | 95.1 | 15 |
| streptomycin | 5 | 60.4 | 28 |
| Tetracycline | | | |
| America | 4 | 672 | 7 |
| Mexico | 4 | 653 | 2 |
| The national names after the antibiotics indicate the country the antibiotics originated in. | | | |
| **Inhibitors of electron transport** | | | |
| dinitrophenol | 4 | 34.3 | 17 |
| FCCP | 19 | 0.20 | 36 |
| potassium cyanide | 17 | 12.2 | 48 |
| sodium azide | 17 | 630 | 19 |

| | n | Ave. | Var. | | n | Ave. | Var. |
|---|---|---|---|---|---|---|---|
| **Herbicides continuation** | | | | **Alkaloids** | | | |
| Metribuzin | 6 | >1200 | | atropine | 9 | 191 | 11 |
| Naproamide | 10 | 289 | 43 | caffeine | 3 | 3700 | 18 |
| Nicosulfuron | 10 | 267 | 11 | immitine | 11 | 122 | 8 |
| Norflurazon | 8 | 182 | 14 | nicotine | 13 | 990 | 13 |
| Oxadiazon | 6 | 269 | 16 | quinine | 4 | 131 | 14 |
| Quniclorac | 6 | >1200 | | qunidine | 4 | 137 | 15 |
| Sethoxydim | 6 | 2.70 | 17 | scopolomine | 5 | 129 | 5 |
| Thiazopyr | 6 | 43.2 | 4 | | | | |
| Thifensulfuron | 7 | 928 | 4 | | | | |
| Trifluralin | 7 | 10.3 | 18 | | | | |

n = the number of samples tests; ave. = the average of the values obtained, reported as part per million (ppm), mg per liter; var. = the variance in the results, the standard deviation divided by the mean.

## 15.2.2.5 Comparisons with other tests

The *Rhizobium* test has been compared with other tests. This is done by finding values in the literature for various toxins using different tests and plotting the log of these values vs. the log of values from the second assay. A regression line was plotted and the correlation coefficient calculated from the regression coefficient (the correlation coefficient is the square root of the regression coefficient). In Figure 15.2.2.2, values for chemicals from two laboratories

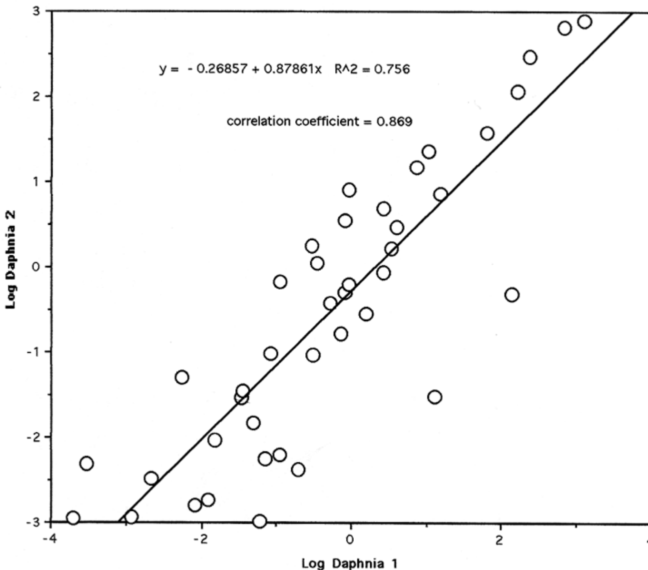


Figure 15.2.2.2. Representative plot of the comparison of two assays. These are data from *Daphnia* 1 (Calleja et al., 1993) and *Daphnia* 2 (Lilius et al.,1994).

(Calleja et al., 1993; Lilius et al., 1994) using the assay involving the sand flea *Daphnia magna* are presented. If two systems give the same results, the correlation coefficient will be 1.00. In the literature there are reports of the toxicity of 50 MEIC chemicals (MEIC, multicenter evaluation of cytotoxicity) tests used in Europe to evaluate different tests for toxicity. When these two tests with *Daphnia* were compared, a correlation coefficient of 0.895 was obtained (Figure 15.2.2.2). This indicates that when a standardized test is run in two laboratories with different personnel, the results are not identical. This discrepancy is noted in tests involving animals. Apparently variations in the strains of animals, differences in care and other factors influence the results of toxicity tests with animals. Often variations of 10x are observed.

This sort of analysis was carried using the *Rhizobium* assay. Published values for the chemicals using Microtox, LD50 reports for rats, IC50 (testing with animal cells), *Daphnia magna*, and HLD (Human Lethal Dose determined from autopsy reports) are included (Table 15.2.2.2). These values for the assay with *Rhizobium* will permit comparisons to be made (Table 15.2.2.3). It should be noted that the values for rats and HLD are much higher than with the other methods. With animal tests, the animals are force fed the chemical, it is injected through a tube into their stomach. Occasionally the toxin is injected intraperitoneally. It is uncertain what happens to the toxic chemical in the acidic stomach. This methodology has been criticized because humans are rarely exposed to toxic chemicals in this fashion. HLD data comes from autopsy reports and it can never be certain how much of the chemical the victim actually came in contact with, it can only be estimated. And this method only provides data for chemicals already in the environment, it is not a good method to predict toxicity.

**Table 15.2.2.2. MEIC chemicals tested. Comparison**

| Chemical tested | Rhizobium, m moles | Microtox, m moles | Rat, m moles | IC50, m moles | Daphnia, m moles | HLD, m moles | rat hepat, m moles |
|---|---|---|---|---|---|---|---|
| acetomenaphen | 1.649 | 2.19 | 15.8 | 1.45 | 0.269 | 1.698 | 10.75 |
| acetylsalicylic acid | 1.106 | 0.145 | 1.122 | 1.7 | 0.932 | 2.818 | 2.662 |
| amitriptyline | 0.0146 | 0.078 | 1.148 | 0.1 | 0.02 | 0.2 | 0.07 |
| barium chloride | 0.109 | | 1.349 | | 0.794 | | 0.47 |
| caffeine | 1.02 | 3.388 | 1 | 1.58 | 3.388 | 1 | 1.596 |
| carbon tetrachloride | 1.91 | 4.786 | 15.1 | 22.1 | 126 | 0.447 | 3.984 |
| chloroform | 5.29 | 12.9 | 7.586 | | 2.63 | 1.738 | 6.198 |
| chlororamphenicol | 4.332 | 1.122 | 7.7 | 0.54 | 5.248 | | 0.402 |
| copper II sulfate | 0.007 | 0.01 | 1.86 | | 0.001 | 0.316 | 0.048 |
| dichloromethane | 4.122 | 37.1 | 18.6 | | 10.5 | 4.17 | 109.1 |
| digoxin | 0.231 | | 0.426 | 0.12 | 12.8 | 0.0014 | 0.271 |
| ethanol | 1643 | 691 | 151 | 158 | 234 | 109.6 | 451 |
| ethylene glycol* | 3427 | 1778 | 75.8 | 322 | 1202 | 25.7 | 358 |

| Chemical tested | Rhizobium, m moles | Microtox, m moles | Rat, m moles | IC50, m moles | Daphnia, m moles | HLD, m moles | rat hepat, m moles |
|---|---|---|---|---|---|---|---|
| hexachlorophene | 0.0002 | 0.019 | 0.138 | 0.0068 | 0.00027 | | 0.002 |
| isopropyl alcohol | 950 | 380 | 83.2 | 90.5 | 155 | 41.7 | 304 |
| iron II sulfate | 0.32 | 0.782 | 2.089 | | 0.302 | 3.020 | 1.621 |
| lindane | 0.141 | 21.9 | 0.263 | 0.36 | 0.0056 | | 0.144 |
| malithion | 0.112 | 0.479 | 0.871 | | | 0.741 | |
| mercury II chloride | 0.00006 | 0.0002 | 3.715 | | 0.00013 | 0.107 | 0.003 |
| methanol | 2130 | 912 | 178 | 673 | 661 | 52.5 | 906 |
| nicotine | 6.1 | 0.224 | 0.309 | 4.52 | 0.0.23 | 0.11 | 3.581 |
| orphenadrine HCI* | 1.168 | 0.005 | 0.832 | | 0.033 | 0.098 | 0.114 |
| paraquat | 0.262 | 2.344 | 0.224 | | 0.1 | 0.166 | 1.176 |
| pentachlorophenol | 0.00054 | 0.02 | 0.1 | 0.025 | 0.0022 | 0.098 | 0.05 |
| phenol | 13 | 0.186 | 3.39 | 4.25 | 0.077 | 1.48 | 0.797 |
| potassium chloride | 290 | 493 | 34.6 | | 490 | 0.288 | 92.22 |
| potassium cyanide | 0.225 | 0.275 | 0.0776 | 1 | 0.0141 | 0.437 | 0.783 |
| quinidine sulfate | 0.422 | 1.202 | 0.617 | 0.036 | 0.0797 | | 0.129 |
| sodium chloride | 288 | 562 | 51.3 | | 60.3 | 17 | 102 |
| sodium oxalate | >13 | 5.428 | | | | | 0.582 |
| theophylline | 1.926 | 13.8 | 1.349 | | 2.63 | 0.724 | 2.175 |
| 1,1,1-trichloroethane | 0.583 | 0.342 | 77.6 | | 6.71 | 1.48 | |
| warfarin | 1.781 | 0.209 | 0.005 | 0.67 | 1.549 | 0.0219 | 0.139 |
| xylene | 1.24 | 0.079 | 34.6 | | 0.851 | 0.468 | 17.47 |

$M_w$ = molecular weight of the compound; n = number of times compound was tested; var = variation in the results, the standard deviation /mean\ toxicity reported as mmoles toxin for the IC50 for the test. The rat test is an LD50. Rhizobium data are from this work. Microtox™, HLD, and Daphnia data are from Calleja, (1993). The IC50 data (pooled data from animal cell tests) is from Halle et al., 1993. Rat hepatocyte data is from Shrivastava (1992). The IC50 samples include only 20 chemicals. The other methods involve about 34 chemicals.

In Table 15.2.2.3 the results of comparisons among these tests are summarized. The data from these 6 determinations were plotted, one assay versus another assay, the regression coefficient noted and the correlation coefficient calculated. The *Rhizobium* assay was the most sensitive for 12 of 33 chemicals. The Microtox assay was most sensitive for 6 of the compounds. The IC50 assay was most sensitive for 6 of the compounds. The *Daphnia* test was most sensitive to 10 of the compounds. The average values for the toxicity of the chemicals was lowest for the IC50, but then it was also the test with the fewest values included.

**Table 15.2.2.3. Comparisons for involving *Rhizobium*, Microtox™, HLD, IC50, *Daphnia magna* and rats**

| | n | $r^2$ | | n | $r^2$ |
|---|---|---|---|---|---|
| Rhizobium x Microtox™ | 34 | 0.875 | Rats x IC50 | 19 | 0.724 |
| Rhizobium x rats | 34 | 0.603 | Rats x HLD | 27 | 0.817 |
| Rhizobium x IC50 | 20 | 0.906 | Rats x Daphnia | 32 | 0.685 |
| Rhizobium x HLD | 34 | 0.628 | Rats x rat hepatocytes | 34 | 0.748 |
| Rhizobium x Daphnia | 34 | 0.888 | IC50 x HLD | 16 | 0.732 |
| Rhizobium x rat hepatocytes | 34 | 0.764 | IC50 x Daphnia | 29 | 0.879 |
| Microtox x rats | 34 | 0.571 | IC50 x rat hepatocytes | 20 | 0.700 |
| Microtox x IC50 | 24 | 0.840 | HLD x rat hepatocytes | 26 | 0.648 |
| Microtox x HLD | 31 | 0.728 | HLD x Daphnia | 29 | 0.702 |
| Microtox x rat hepatocytes | 33 | 0.700 | Daphnia x rat hepatocytes | 33 | 0.814 |
| Microtox x Daphnia | 34 | 0.846 | | | |

n = number of samples in the test. One test was compared with another, the log of the values plotted, a regression line fit and the correlation coefficient determined from the regression coefficient, $r^2$.
The data for rats, Daphnia, HLD is from Calleja et al., 1994. The data for IC50 is from Halle et al., 1992. The data for rat hepatocytes is from Shrivastava et al., 1992.

In Table 15.2.2.4, the results of these comparisons with the results of the *Rhizobium* test are presented. The *Rhizobium* test correlates well with Microtox™, *Daphnia*, and particularly well with the 20 samples in the IC50 test. The comparison of the *Rhizobium* assays with other published results has been examined in detail (Botsford, 2000a). The *Rhizobium* test has been compared with values from several laboratories for the Microtox™ assay and correlation coefficients have varied from 0.750 to 0.893 indicating the two methods provide comparable results. Four comparisons with values for *Daphnia* have provided correlation coefficients from 0.776 to 0.953 indicating that the *Rhizobium* assay agrees well with *Daphnia*.

QSAR provides a method to predict the toxicity of a compound from the structure of the compound and the water/octanol partition coefficient (Nirmilkhandan and Speece, 1988). This method has been compared with assays using sewage sludge and Polytox™ (Sun et al., 1993). The method compares well with both these other techniques. An examination of the data provided by tests for toxic chemicals using these techniques suggested that they are not as sensitive as more direct methods for determining toxicity. It was found that Microtox™, rat hepatocytes, the *Rhizobium* assay, and *Daphnia* all provided more sensitivity to toxic chemicals than did the QSAR estimates (Botsford, 2000a). The IC50 values for these other techniques were lower than those computed using the QSAR method. QSAR data are not often found in the literature.

**Table 15.2.2.4. The *Rhizobium* assay compared with other assay methods**

| | n | cc | Reference |
|---|---|---|---|
| 3T3 cells x Rhizobium | 9 | 0.908 | Ekwall et al., (1989) |
| Asictes cells x Rhizobium | 34 | 0.870 | Romert et al., (1994) |
| Biotox™ x Rhizobium | 24 | 0.928 | Kahru and Bordchardt (1994) |
| B. subtilis x Rhizobium | 20 | 0.619 | Kherzmann (1993) |
| Daphnia 1 x Rhizobium | 35 | 0.891 | Calleja et al., (1993) |
| Daphnia 2 x Rhizobium | 35 | 0.897 | Lilius et al., (1994) |
| pooled Daphnia x Rhizobium | 14 | 0.775 | Calleja, Liilius, Munkitrick |
| E. coli x Rhizobium | 20 | 0.641 | Kherzman (1993) |
| fathead minnow x Rhizobium | 15 | 0.942 | Munkitrick et al., (1991) |
| guppies x Rhizobium | 9 | 0.950 | Konemann (1981) |
| HepG2 cells x Rhizobium | 9 | 0.892 | Ekwali et al., (1989) |
| Microtox™ x Rhizobum | 35 | 0.803 | Kaiser and Palabrica (1991) |
| Microtox™ x Rhizobium | 15 | 0.758 | Munkittrick et al., (1991) |
| mice x Rhizobium | 35 | 0.651 | Calleja et al., (1991) |
| Polytox™ 1 x Rhizobium | 16 | 0.903 | Sun et al., (1993) |
| Polytox™ 2 x Rhizobium | 15 | 0.796 | Elanabarwy et al., (1988) |
| sludge x Rhizobium | 16 | 0.853 | Sun et al., (1993) |
| trout hepatocytes x Rhizobium | 35 | 0.760 | Lilius et al. (1994) |
| trout figerlingx x Rhizobium | 15 | 0.910 | Munkittrick et al., (1991) |
| QSAR x Rhizobium | 19 | 0.779 | Sun (1994) |
| pooled minnow x Rhizobium | 32 | 0.808 | Geiger et al., (1991), Munkitrick et al., (1991) |

n = number of samples in the comparison; cc = the correlation coefficient, the square root of the regression coefficient.

### 15.2.2.6 Toxic herbicides

When the toxic herbicides were studied, it was found that the animal tests supplied by the manufacturers indicated that most of the herbicides were not toxic. The tests run by the manufacturers with trout fingerlings and with *Daphnia* indicated that most of the herbicides were quite toxic. The *Rhizobium* work also showed that most of the herbicides were toxic at levels lower than 1000 ppm. The trout fingerlings showed all but one of the herbicides was toxic at this level and *Daphnia* indicated that all but 2 were toxic (Hillaker, 1998). This shows the necessity of running "batteries of tests," multiple tests with a compound. Every method of determining toxicity has some chemicals it cannot detect as toxic and some chemicals that are detected at very low levels. For example, the *Rhizobium* assay does not detect phenol as being very toxic but detects pentachlorophenol as being extremely toxic,

the test is at least an order of magnitude more sensitive to pentachlorophenol than any other test found.

### 15.2.2.7 Toxicity of divalent cations

Reduction of the dye is inhibited by divalent cations in the *Rhizobium* system. Common ions, calcium and magnesium, inhibit the reduction of the dye. The toxicity of the ions is shown in Table 15.2.2.5. Mercury and cadmium, generally thought to be the most toxic minerals were the most toxic with this assay. Calcium and magnesium are also toxic. Water and soil samples typically contain calcium and magnesium so in order to analyze water and soil samples for toxic organic chemicals, a method to eliminate this inhibition by metal ions was sought.

**Table 15.2.2.5. Toxicity of divalent cations**

| Minerals | n | var. | m moles | Minerals | n | var. | m moles |
|---|---|---|---|---|---|---|---|
| $Ba^{+2}$ | 6 | 33 | 0.109 | $Mg^{+2}$ | 9 | 20 | 0.404 |
| $Cd^{+2}$ | 12 | 41 | 0.004 | $Mn^{+2}$ | 11 | 35 | 0.045 |
| $Ca^{+2}$ | 11 | 33 | 0.05 | $Hg^{+2}$ | 10 | 23 | 0.0006 |
| $Co^{+2}$ | 11 | 46 | 0.009 | $Ni^{+2}$ | 11 | 11 | 0.452 |
| $Cu^{+2}$ | 13 | 19 | 0.007 | $Se^{+2}$ | 9 | 39 | 1.849 |
| $Fe^{+2}$ | 7 | 18 | 0.587 | $Zn^{+2}$ | 10 | 7 | 0.062 |
| $Fe^{+3}$ | 14 | 27 | 0.098 | | | | |

n = number of samples tested; var. = variation, standard deviation divided by the mean. Values reported as m moles $l^{-1}$. All minerals except ferrous ion as chloride salts. Several were tested as both chlorides and sulfates with little difference. When ferrous sulfate was tested, it was made up fresh each day before the assay.

EDTA is used routinely in biochemistry to chelate divalent metal ions. It was thought this might chelate the calcium and remove it from the system. EDDA and EGTA are also used and are thought to chelate calcium more effectively than EDTA. Neither of these chelators affected the reaction, both were simply slightly toxic (about 3000 ppm). A series of experiments were run and it was found that the inhibition of reduction caused by 1.4 to 1.6 µmoles of calcium was relieved by 1 µmoles EDTA. There is not a stoichiometric relationship between EDTA and the metal ion. It is not simply chelating the metal ion.

It was found that 2.5 µmoles EDTA would eliminate the toxicity of all the ions at their IC50, the concentration of cation that inhibited reduction of the dye 50%. Thus 2.5 µmoles EDTA would eliminate toxicity from 5.5 µmoles calcium but only 0.006 µmoles mercury. It was observed initially that the toxicity of most organic chemicals could be determined in the presence of 2.5 µmoles EDTA. This suggested there could be two mechanisms involved in the reduction of MTT. One is inhibited by toxic organic chemicals and the second is inhibited by divalent cations.

### 15.2.2.8 Toxicity of organics in the presence of EDTA

A series of experiments were run looking at the toxicity of organic chemicals in the presence of 0.74 µmoles calcium (25 ppm, this concentration inhibits reduction of the dye completely) and 2.5 µmoles EDTA. Four of the 35 chemicals tested had greater toxicity with EDTA and calcium than in the controls. Two chemicals were no longer toxic. The toxicity of 16 of the chemicals was not affected by the calcium and EDTA. The toxicity of 13 chemicals was decreased by at least 10% but was not eliminated by the addition of EDTA and cal-

cium. The toxicity of 4 chemicals was enhanced, was greater, when the calcium and EDTA were present.

The addition of calcium and EDTA at these concentrations had no effect on the apparent toxicity of: 1,4-dinitrobenzene, the herbicide 2,4-D, 2,4-dinitrotoluene, 4-chloro-benzoate, carbon tetrachloride, chloroform, cynazine, hexachlorophene, isonicotinic acid the insecticide Lindane, o-nitrophenol, p-toluidine, the antibiotic Streptomycin, tetrachloroethylene and trichlorophenol.

The addition of calcium and EDTA reduced the toxicity at least 10% of 2,6-dinitrocresol, 2,4-dinitrophenol, 2,6-dinitrophenol, 2,6-dinitrotoluene, 2-methyl resourcinol, 3-phenoxybenzoate, 2,4-dinitrocresol, the detergent CTAB, the antibiotic Neomycin, p-hydroxybenzoate, pentachlorophenol, salicylic acid, and trichloroethylene.

p-amino benzoic acid and p-hydroxy benzoate were no longer toxic with these levels of calcium and EDTA.

3-chlorobenzoate, 3-methyl-4-nitro-phenol, and the detergents sodium lauryl sulfate and Tween 80 had increased toxicity with the addition of calcium and EDTA. No correlation between the structure of the chemical and the effect of EDTA and calcium could be ascertained. For example, streptomycin and neomycin are both amino glycoside antibiotics. Calcium and EDTA did not affect the toxicity of streptomycin but nearly eliminated the toxicity of neomycin.

Several chemicals were tested with 25 μmoles EDTA and 7.4 μmoles of calcium, 10 times the amount used in the experiments reported. The results of this experiment are shown in Table 15.2.2.6. In the presence of high levels of EDTA and calcium, isonicotinic acid is no longer toxic. Dinitrophenol and 3-phenoxy benzoate, in the presence of low levels of EDTA and calcium were not as toxic, in the presence of high levels, it had the same toxicity. In presence of low levels of EDTA and calcium, Streptomycin was comparably toxic, the calcium and EDTA did not affect the toxicity. In the presence of high levels, Streptomycin was extremely toxic. Pentachlorophenol, a common soil contaminant, had reduced toxicity with low levels of EDTA and calcium, had elevated levels of toxicity with high levels of EDTA and calcium. This work shows that the effect of EDTA and specific toxins must be worked out before any conclusions as to the toxicity of the compound in the presence of EDTA and calcium can be established.

Calcium and magnesium are commonly found in water. Obviously if this assay is to be used with water samples, EDTA must be added. It must be determined using water uncontaminated with organic toxins how much EDTA must be used to compensate for the divalent cations. Often the concentration of divalent cations is determined by atomic absorption spectroscopy. However, these values do not agree with the toxicity relieved by EDTA. Soil samples with as much as 5 gm calcium (45 mM) per kg soil have been assayed using 2.5 μmoles EDTA in each sample (Hillaker, 1996). The calcium is complexed with sulfate and phosphate ions and the calcium is not available to the cell, is not seen by the mechanism that reduces the dye. Levels of soluble calcium and magnesium in water are very low. We have found that 2.5 μmoles of EDTA relieves the inhibition caused by divalent cations in all water and soil samples tested thus far (Botsford, 2000b).

**Table 15.2.2.6. Effect of high concentrations of EDTR and calcium on toxicity (Inhibition in %)**

| Compound | 2.5 μmole EDTA, 0.74 μmole Calcium | | 25 μmole EDTA, 7.4 μmole Calcium | |
|---|---|---|---|---|
| | Control | + EDTA, + Ca | Control | + EDTA, + Ca |
| isonicotinic acid | 27.0 | 25.4 | 30.9 | 105 |
| 2,4-dinitrophenol | 44.7 | 63.0 | 58.2 | 50.0 |
| Streptomycin | 55.0 | 48.9 | 47.2 | 0.00 |
| Neomycin | 0.037 | 62.4 | 77.2 | 77.3 |
| pentachlorophenol | 48.0 | 63.7 | 26.0 | 13.3 |
| 3-phenoxybenzoate | 54.3 | 74.0 | 34.5 | 37.8 |

### 15.2.2.9 Mechanism for reduction of the dye

It is not known how the dye is reduced. It is not known why toxic chemicals inhibit the reduction. It is thought that tetrazolium dyes are reduced by cytochromes (Altman, 1975). But this has been questioned (Marshall et al., 1993). In eucaryotic cells, all the cytochromes are in the mitochondria. Marshall's group has found that the dyes are reduced in preparations from cells with the mitochondria removed. It has been found that a mutant of *Escherichia coli* lacking one of two major cytochromes found in this bacterium is unable to reduce MTT (Botsford, unpublished). But in *E. coli*, reduction of the dye is not inhibited by toxic chemicals. This may not be an analogous situation. The dye could be reduced by a different mechanism.

*R. meliloti*, like most other bacteria, has many reductases. Some of these are membrane associated and damage to the membrane could affect the reductase. One of these reductases could be responsible for reduction of the dye. It has been found that the MTT is transported into the cell before it is reduced. The reduced dye is inside the cells. Cells with the dye can be concentrated by centrifugation, the dye appears in the cell pellet. None of the dye is in the supernatant. Toxic chemicals could interfere with the transport of dye into the cell prior to reduction.

Transposon insertion mutants unable to reduce the dye have been obtained and five mutants have been isolated. All grow very slowly in minimal media supplement with 0.1 % casamino acids and obviously all have lost a critical function. With these mutants it should be possible to clone and then to sequence the function responsible for reduction of the dye. From the sequence, the nature of the function can be determined.

In our studies comparing the *Rhizobium* assay with other assays, it was observed that tests using viable animals were almost always less sensitive to toxins. Tests using *Daphnia*, the various animal cell tests and Microtox™ and the *Rhizobium* test seemed to be most sensitive. Tests using fish (fathead minnow, trout fingerlings) give results comparable to tests with the bacterial indicators. Polytox™ and QSAR were less sensitive, had higher IC50 values, but were more sensitive than the tests using viable animals. Were the author asked to recommend a test procedure to indicate if a chemical were toxic, the author would recommend an animal cell test, probably using freshly isolated rat liver hepatocytes, the *Rhizobium* test and the Microtox™ test. Tests with freshly isolated rat hepatocytes would not require that the cells be grown in a laboratory situation and this would be much simpler. These three procedures are much more sensitive than tests involved live animals. These three tests would be simpler than tests with *Daphnia*. This would provide a "battery of

tests", would not offend animal rights advocates and should indicate if the chemical is dangerous. All could be performed by personnel with chemical laboratory skills.

### 15.2.2.10 Summary

This work shows that the *Rhizobium* test provides results comparable to other tests. Tests seem particularly comparable to work with *Daphnia magna* and with results from *in vitro* tests with animal cells. The test is simple, unskilled laboratory workers can master it quickly. The test is inexpensive, no specialized equipment is required, given cells, any laboratory able to carry out simple chemical analysis should be able to perform the assay. The test is rapid, a sample can be tested and analyzed in an hour, the test does not take several days. It offers an ideal first test for toxic chemicals (Blaise, 1991)

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## 15.2.3 DESCRIPTION OF AN INNOVATIVE GC METHOD TO ASSESS THE INFLUENCE OF CRYSTAL TEXTURE AND DRYING CONDITIONS ON RESIDUAL SOLVENT CONTENT IN PHARMACEUTICAL PRODUCTS

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The presence of residual solvents (RS) in pharmaceutical substances occurs for various reasons. Solvents are involved in all steps of raw material synthesis and pharmaceutical productions. The search for the presence of RS in a pharmaceutical product and their concentrations are now mandatory in any new monographs (as detailed in Chapter 16.2).

The RS remaining in the crystals of pharmaceutical products may be the cause of health disorders because, when a drug is taken every day, chronic toxicity may occur.

The presence of RS may have other consequences, such as modifying stability, organoleptic characters, pharmacotechnical parameters (flow properties, crystalline form, compression ability) and biopharmaceutical characteristics, that may fluctuate according to RS content (as detailed in chapters 14.21.1 and 16.2). It is therefore necessary to reduce the residual solvents contained in crystalline particles as much as possible.

It is well known that solvents can exist in three different states within the crystals:

- Solvents adsorbed on the crystal faces: these are generally easily desorbed during conventional drying because the binding forces between solvents and crystals are very weak.
- Occluded solvents such as microdroplets in the crystal: these are often difficult to extract. Generally, they can escape when the crystal is being dissociated:
  - during grinding, potentially leading to clodding,
  - during storage leading to very compact aggregates.
- Solvents bound to drug molecules in the crystal and known as "solvates". These bound solvents escape at a characteristic temperature, producing desolvated forms; the solvate and the desolvated forms are two different crystalline entities that can exhibit very different mechanical behaviors.

The main objective of any chemist crystallizing pharmaceutical raw materials should be either the total elimination of the organic solvents or the significant reduction of RS level in order to be below the regulatory limits.[1]

### 15.2.3.1 Description of the RS determination method

There are several analytical methods to assess the RS content of drugs. Among them, the gas chromatography (GC) is largely preferred. We refer to the chapter 16.2 for more details. In direct injection methods, the products in which RS are included are usually dissolved in an appropriate solvent and then directly injected into a gas chromatograph (GC). The main problem with these techniques is that non-volatile substances are gradually retained in the column, causing a rapid decrease in its sensitivity and efficiency; this is one of the reasons why headspace techniques are increasingly used instead.

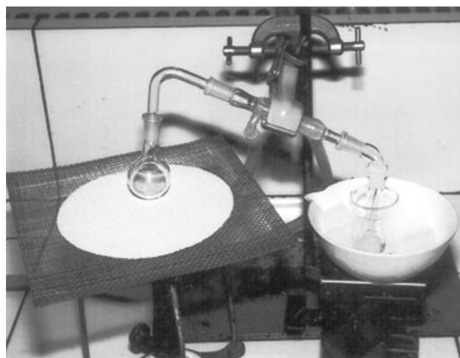

Figure 15.2.3.1. Photograph of the micro-distilling device.

To avoid these problems, we have developed a method consisting initially of the complete dissolution of the substance in an appropriate solvent followed by micro-distillation and then finally of the injection of the distillate into the GC. This technique allows for the complete recovery of RS without the drawbacks mentioned above.[2,3]

The operating conditions are as follows: the powders to be analyzed are poured into a micro-distilling flask. An appropriate solvent is chosen to allow the product as well as the RS to be completely dissolved. The solvent should not interfere with the RS extracted; for example, in the following studied cases: methanol for dioxane, chloroform, tetrahydrofuran, hexane and dichloromethane determinations and 1-butanol for ethanol determination. Assay solutions were obtained by completely dissolving 500 mg of the product to be tested in 5 ml of the appropriate solvent. The micro-distillation is carried out until complete dryness and is followed by condensation of the solvent vapor. The distillate is collected in a gauge glass set on ice. The assembly of the system is photographed on Figure 15.2.3.1.

In this way the non-volatile substances stay entrapped in the micro-distilling flask and the totality of the RS are recovered. The distillate is then directly injected into the GC. The method was validated (specificity, linearity, repeatability, reproducibility...) by distilling, in the same operating conditions, defined standard solutions of solvents. The recovery of RS is very good as can be seen in the example reported on Table 15.2.3.1.

### 15.2.3.2 Application: Influence of crystal texture and drying conditions on RS content

After washing the crystals, drying is the most effective way of lowering the content of organic volatile impurities. The required drying conditions differ greatly according to the solvent state in the crystalline particles, to the thermodynamic events that can occur when the substance is heated, and also to the texture of these particles.

**Table 15.2.3.1. Example of a calibration curve and recovery calculations of dioxane distilled in methanol**

| Dioxane standards | | Dioxane recovered after distillation | |
|---|---|---|---|
| | area under curve and variation coefficient (%) | area under curve and variation coefficient (%) | % and ppm recovered |
| 44.8 ppm | 17161.0±705.5, v.c: 4.11 % | 16819.3±631.5, v.c: 3.75 % | 98.01 %, 43.9 ppm |
| 56 ppm | 25904.7±1493.1, v.c: 5.76 % | 25869.0±748.4, v.c: 2.89 % | 99.86 %, 55.9 ppm |
| 112 ppm | 66147.3±1010.4, v.c: 1.53 % | 66831.7±472.6, v.c: 0.71 % | 101.03 %, 113.2 ppm |
| 168 ppm | 108157.7±2026.6, v.c: 1.53 % | 107361.0±1546.4, v.c: 1.44 % | 99.26 %, 166.8 ppm |
| 224 ppm | 146674.0±1490.4, v.c: 1.02 % | 146722.7±2469.7, v.c: 1.68 % | 100.03 %, 224.1 ppm |
| linear regression | Y = 724.74 X - 14855.6 | Y = 724.29 X - 14889.7 | mean : 99.64±1.11 % v.c : 1.1 % |
| correlation coeff. | 0.9999 | 0.9999 | |

Among all parameters influencing RS content, crystal texture is of utmost importance. It is evident that a crystal exhibiting a porous texture will enable the easy escape of a solvent while a compact and dense crystal will retain the solvent inside its structure whatever the type of particles: monocrystalline (i.e., monoparticular) or "polycrystalline". The term "polycrystalline" particles will be employed to designate elementary particles that can be composed of agglomerates, spherolites or "spherical crystals" according to Kawashima.[4]

To illustrate the importance of the texture of particles on RS content, we can consider some examples taken from our laboratory experiments. Several crystallization processes were investigated on pharmaceutical products with different solvents leading either to compact monocrystalline particles or "sintered-like" (i.e., microcrystallites fitted into each other and partially welded involving a porous texture) or polycrystalline particles which were more or less dense.[2,3] The physical study of these particles such as, optical microscopy, particle size analysis, electron scanning microscopy and thermal analysis, have been used to link the RS contents with the drying conditions of crystals and to demonstrate that the optimal drying conditions differ greatly according to the texture of the particles.

As we shall see in a first example of monocrystalline particles of paracetamol recrystallized in dioxane, a wide open texture is generally favorable to a low RS content after a progressive drying at a moderate temperature to avoid the formation of a superficial crust. In fact, in this case, the drying conditions of crystals highly influence their residual solvent content. Optimal drying conditions seem to be the progressive and moderate ones. In contrast, too drastic drying conditions may hinder the solvent escape by a "crust" effect. This crust is due to a drying temperature which is too high, leading to a melt and a dissolution of the surface of the crystals. Finally, when a desolvation occurs during the drying, it can modify the texture of crystals and form a crust. The intensity of the phenomenon depends on the solvent.

To remove the solvents efficiently, it is then necessary to exceed the desolvation temperature of the solvates that may be produced during crystallization.

With this example we can clearly point out that the crystal texture is a determining factor in the complete escape of the solvent: when the crystalline texture is sintered-like, after the desolvation of solvate crystals, progressive drying is necessary to prevent the "crusting" phenomenon.

The drying conditions should also be adapted to the area offered to the evaporation of solvent, particularly in the case of polycrystalline particles presenting a high porosity and a large surface to be dried. This important surface is due to the disordered rearrangement of very small crystals inside the particles. To illustrate this, we shall see in a second example the cases of spherical crystals of meprobamate and ibuprofen agglomerates. In both cases the RS can escape easily from the large surface of polycrystalline particles.

Lastly, we shall see in a third example, that we must not forget that thermodynamic phenomenon can occur under drying. This is a very particular example because the pharmaceutical product used (paracetamol) presents a polymorphic transition that can occur during the drying phase and lead to a new organization inside the crystal, allowing the escape of the RS. This can be very interesting in the case of products with a low transition temperature. The usual polymorphic form of commercialized paracetamol is the monoclinic form; but in particular cases small amount of metastable orthorhombic form can be obtained. In the case of paracetamol, at the transition temperature (156°C) we observe a brutal solvent escape that could be due to the solid-solid transition undergone by the orthorhombic form into the monoclinic one. The disorder produced and the increase of the specific volume occurring during this first order transition allow the occluded solvents to escape more easily.

### *15.2.3.2.1 First example: monocrystalline particles of paracetamol*

Preparation of monocrystalline particles

In this first example, paracetamol was recrystallized separately in three different types of solvents (ethanol, water and dioxane) with different boiling points, molecular weights, dielectric constants and paracetamol solubilising power. After the crystallization process,[5] crystals were separated by filtration under vacuum and washed with the same crystallization solvent. Each batch was divided into four fractions to be dried differently.

Particle drying conditions

The paracetamol crystals were submitted to four different drying conditions[2] (Table 15.2.3.2):

Drastic: under vacuum at 100°C for 3 hours.

Drastic: in a ventilated oven at 100°C for 3 hours.

Progressive: in a ventilated oven at 60°C for 1 hour, at 80°C for 1 hour and finally at 100°C for 1 hour.

Very moderate: under ventilated hood at 20°C for 24 hours and then in a ventilated oven at 35°C for 48 hours.

Morphological aspect of the particles

Dried crystals were observed by optical and scanning electron microscopy (SEM) in order to measure their Ferret mean diameter and to determine their habit and texture. The mean diameters of crystals obtained from different solvents and submitted to different drying conditions are reported in Table 15.2.3.3.

**Table 15.2.3.2. Drying conditions studied on the different particles**

| Type of particles | Drugs | Type of drying | Temperature | Time |
|---|---|---|---|---|
| monocrystalline | paracetamol | drastic under vacuum | 100°C | 3 h |
| | | drastic under ventilation | 100°C | 3 h |
| | | progressive | 60°C<br>+ 80°C<br>+ 100°C | 1 h<br>+ 1 h<br>+ 1 h |
| | | very moderate | 20°C<br>+ 35°C | 24 h<br>+ 48 h |
| polycrystalline | meprobamate | drastic | 90°C | 2 h |
| | | progressive | 60°C<br>+ 75°C<br>+ 90°C | 30 min<br>+ 30min<br>+ 1 h |
| | ibuprofen | moderate | 60°C | 2 h |
| | | very moderate | 40°C | 2 h |
| | paracetamol | drastic flash | 156°C | 10 min |
| | | drastic flash | 156°C | 30 min |
| | | drastic | 100°C | 2 h |
| | | drastic + flash | 100°C<br>+ 156°C | 2 h<br>+ 10 min |
| | | progressive | 60°C<br>+ 80°C<br>+ 100°C | 30 min<br>+ 30 min<br>+ 1 h |
| | | progressive + flash | 60°C<br>+ 80°C<br>+ 100°C<br>+ 156°C | 30 min<br>+ 30 min<br>+ 1 h<br>+ 10 min |

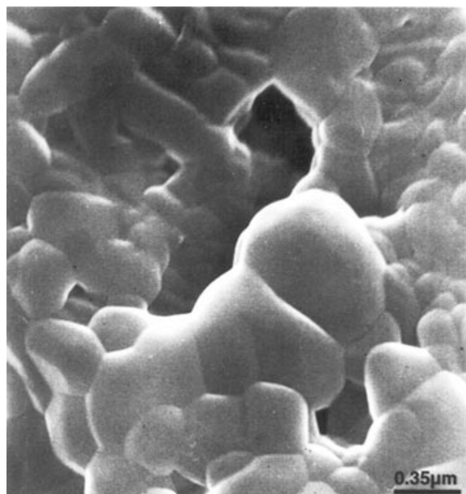


Figure 15.2.3.2. SEM photograph of paracetamol recrystallized in dioxane and submitted to moderate drying (Photograph from reference[2]).

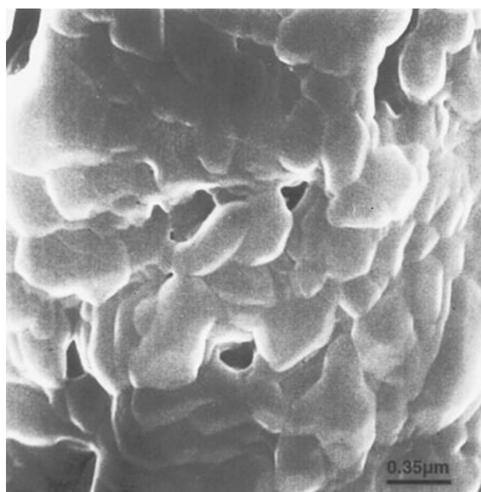


Figure 15.2.3.3. SEM photograph of paracetamol recrystallized in dioxane and submitted to progressive drying (Photograph from reference[2]).

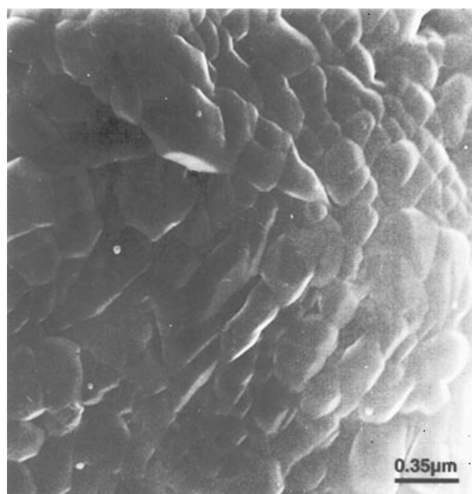


Figure 15.2.3.4. SEM photograph of paracetamol recrystallized in dioxane and submitted to drastic drying (Photograph from reference[2]).

The optical microscopy investigation shows some significant differences in mean diameter and crystal habit due to crystallization conditions.

In the case of water and ethanol, the crystals are transparent and rather regular, their habit is generally parallelepipedal. The only difference to be noted is the mean diameter of these particles; the particles recrystallized in water being 3 times larger. ESM reveals a surface that remains smooth in all drying conditions; their texture seems compact and dense.

In contrast, crystals obtained from dioxane are rather different to those obtained from ethanol and water: they are opaque to transmitted light and their habit is irregular. Their mean diameter is slightly smaller than crystals from ethanol.

It is to be noted that all the crystals obtained from the three solvents present the same polymorphic form: the monoclinic form.

**Table 15.2.3.3: Mean Ferret diameters of particles (Data from references[2,3])**

| Monocrystalline particles | Mean diameter, μm | Polycrystalline particles | Mean diameter, μm |
|---|---|---|---|
| paracetamol / dioxane | 122 | meprobamate spherical crystals | 145 |
| paracetamol / ethanol | 149 | ibuprofen agglomerates | 350 |
| paracetamol / water | 538 | paracetamol spherolites | 113 |

Scanning electron microscopy (SEM) carried out on these crystals reveals different behaviors according to the drying conditions tested. The crystals obtained from dioxane are similar to blocks of sintered particles since they consist of microcrystallites bound to each other, as if partially welded.[5] In fact, the measurement of mercury porosity indicated a very high porosity. This sintered aspect concords with the non-transmission of light through the whole crystal.

In fact, paracetamol forms a solvate with dioxane.[5] Its departure does not modify the crystal habit but the surface can be seen as perforated.

When the drying is very moderate, the crystal surface is perforated by numerous holes through which solvent escapes; these anfractuosities give the impression of a porous sintered-like texture (Figure 15.2.3.2). With progressive drying, the number of holes decreases (Figure 15.2.3.3). On the contrary, when the drying is drastic, the surface is relatively smooth (Figure 15.2.3.4). This can be explained by the too drastic drying conditions involving the formation of a crust at the surface of the crystal preventing the escape of the solvent from the crystal.

Residual solvent determination

Gas Phase Chromatography (for dioxane and ethanol) was performed on a Varian 1440 Chromatograph with a Flame Ionization Detector; packed column Porapack Super Q (Alltech, France), mesh range 80/100, length 1.8 m; internal diameter 2.16 mm; carrier gas was nitrogen (40 ml/min); injector: 210°C; detector: 250°C.

For dioxane: column temperature: isotherm at 170°C. Injection: 10µl. Retention Times (RT): methanol 1.5 min; dioxane 16 min.

For ethanol: column temperature: isotherm at 210°C. Injection: 5µl. RT: ethanol 1.5 min; 1-butanol 4 min.

Determination of residual water on paracetamol crystallized in water: according to the titrimetric direct method of Karl Fischer.

The residual solvent concentrations of the crystals obtained are reported in Table 15.2.3.4.

**Table 15.2.3.4. Residual solvent content of crystals obtained from different solvents and submitted to different drying conditions (Data from reference[2])**

| | Drastic | Drastic under vacuum | Progressive | Moderate |
|---|---|---|---|---|
| Dioxane | 126 ppm | 183 ppm | 25 ppm | 53 ppm |
| Ethanol | 2045 ppm | 2501 ppm | 1495 ppm | 2072 ppm |
| Water | 0.59 % | 0.68 % | 0.51 % | 0.56 % |

Whatever the drying conditions may be, the content of dioxane is always lower than the 380 ppm ICH limit[1,6] (Table 15.2.3.5). Progressive drying always gives lower level residual solvent. In fact, drastic drying, in a ventilated oven and under vacuum, leads to the formation of a superficial “crust” which hinders the solvent escape; this can be clearly visualized on the scanning electron photomicrograph (Figure 15.2.3.4); and can explain the relatively high content of residual solvents.

**Table 15.2.3.5. Solvent class and concentration limits in pharmaceutical products (Data from references[1,6])**

| | Class | Concentration limit, ppm | | Class | Concentration limit, ppm |
|---|---|---|---|---|---|
| Chloroform | 2 | 60 | Hexane | 2 | 290 |
| Dioxane | 2 | 380 | Dichloromethane | 2 | 600 |
| Ethanol | 3 | 5000 | Tetrahydrofuran | 3 | 5000 |

#### *15.2.3.2.2 Second example: polycrystalline particles of meprobamate and ibuprofen*

Preparation of polycrystalline particles

The polycrystalline particles were produced using various crystallization processes and designed to obtain directly compressible particles of pure drug, as tablets cannot be formed by direct compression of the raw materials.

*Spherical crystals of meprobamate*

These spherical particles were prepared following the usual preparation process for spherical crystals described by Guillaume.[7] Spherical crystals of meprobamate appear when stirring a mixture of three liquids in the crystallizer: methanol allowing meprobamate to dissolve; water, as a non-solvent, causing meprobamate precipitation; chloroform as a bridging liquid to gather in its dispersed droplets, meprobamate microcrystallites that finally form “spherical crystals”.

*Ibuprofen agglomerates*

Ibuprofen agglomerates were prepared by a phase separation process in a mixture of ethanol and water (50/50 v/v).[8] The saturated solution obtained at 60°C was constantly stirred and cooled down to room temperature.

Particle drying conditions

Depending on the melting point of the drugs, different drying conditions were applied in a ventilated oven. Drastic and progressive temperature conditions were studied for each type of polycrystalline particles (Table 15.2.3.2).

*Spherical crystals of meprobamate*

Taking into account the melting point of meprobamate (105°C), temperatures higher than 90°C must be avoided. The different drying conditions were: drastic drying at 90°C for 2 hours and progressive drying at 60°C for 30 minutes, then at 75°C for 30 minutes and finally at 90°C for one hour.

*Ibuprofen agglomerates*

This is a particular case; a low drying temperature must be applied because of the very low melting point of ibuprofen (76°C). The two drying conditions studied were: 40°C for two hours or 60°C for two hours.

Morphological aspect of the particles

*Spherical crystals of meprobamate*

Meprobamate crystals appear as more or less rounded opaque particles (Table 15.2.3.3). Their consistence is friable. Particle size distribution is very narrow. SEM photograph of meprobamate crystals shows nearly spherical particles; their surface seems apparently smooth (Figure 15.2.3.5). However, at high magnification, the surface appears to be coated with flat crystals (Figure 15.2.3.6).

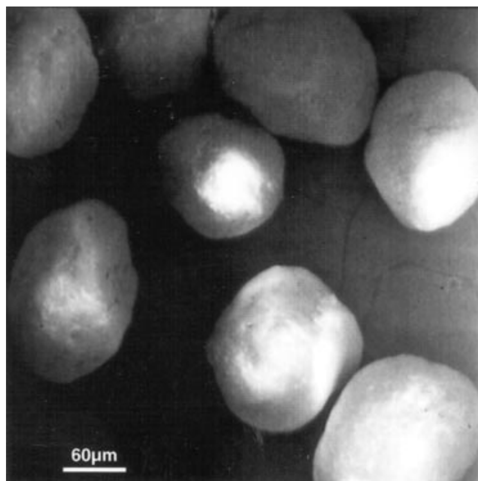


Figure 15.2.3.5. SEM photograph of “spherical crystals” of meprobamate (Photograph from reference[3]).

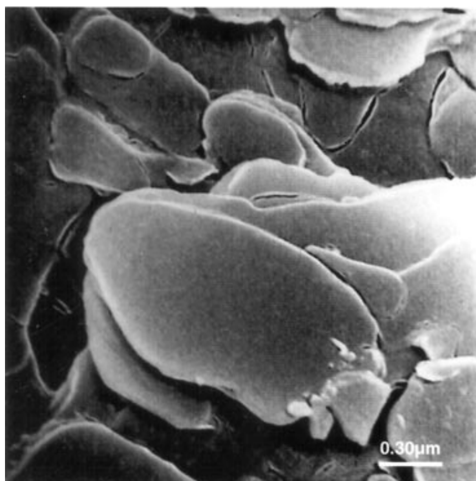


Figure 15.2.3.6. SEM photograph of the surface (high magnification) of “spherical crystals” of meprobamate (Photograph from reference[3]).

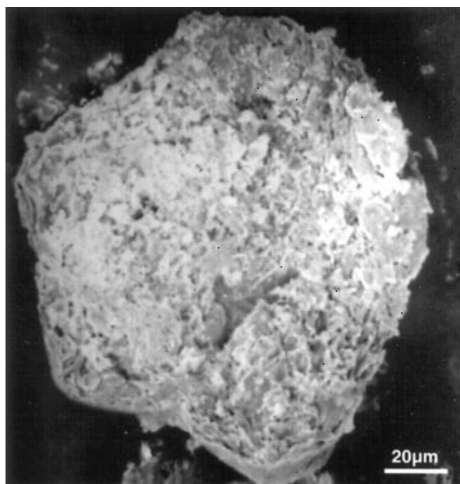


Figure 15.2.3.7. SEM photograph of the cross-section of “spherical crystals” of meprobamate (Photograph from reference[3]).

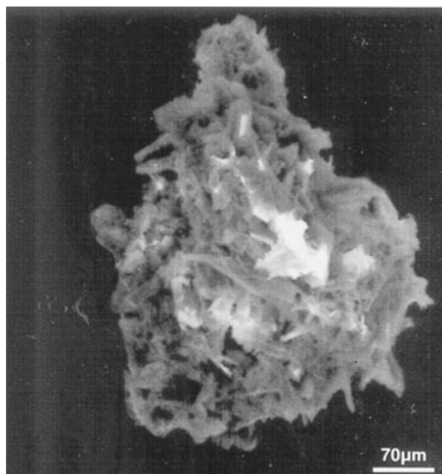


Figure 15.2.3.8. SEM photograph of ibuprofen agglomerates (Photograph from reference[3]).

The cross-section surface shows a dense tangling-up of flat crystals inside the particle (Figure 15.2.3.7). Flat crystals are concentrically disposed at the periphery of the rounded particles, like a shell. The inside of the particle is a disordered arrangement of small flat crystals. Thus, the surface offered to evaporation is very important and this should lead to the easy escape of RS.

*Ibuprofen agglomerates*

Ibuprofen particles are large agglomerates of flat crystals with quite soft consistence and a nearly rounded shape (Table 15.2.3.3).

Ibuprofen crystals observed under SEM are in total disorder (Figure 15.2.3.8). This disordered arrangement of microcrystallites inside the polycrystalline particles gives them a high isotropy of particles arrangement that should improve their compression capacity.

Residual solvent determination

For organic solvents, gas phase chromatography was performed on a Shimadzu GC-14B chromatograph fitted with a Flame Ionization Detector and a CR-6A Shimadzu integrator.

The packed column was Porapack super Q (Alltech, France), mesh range 80/100, length 1.80 m, internal diameter 2.16 mm. Carrier gas: anhydrous nitrogen. Injector temperature: 200°C. Detector temperature: 220°C. The chromatographic conditions were:

- For chloroform in meprobamate crystals: isotherm at 150°C, injection: 5 µl, RT: methanol 0.8 min, chloroform 6.7 min.
- For ethanol in ibuprofen crystals: isotherm at 170°C, injection: 5 µl, RT: ethanol 1.1 min, 1-butanol 5.3 min.

Determination of residual water was carried out using Karl Fischer's titrimetric direct method after calibration with natrium tartrate and dissolution of ibuprofen or meprobamate crystals in methanol.

The residual solvent concentrations of the polycrystalline particles are reported in Tables 15.2.3.6 and 15.2.3.7.

**Table 15.2.3.6. Residual solvent concentration of meprobamate spherical crystals submitted to different drying conditions (Data from reference[3])**

| | Chloroform | Water |
|---|---|---|
| Progressive drying* | 345±22 ppm | 0.71%±0.07% |
| Drastic drying** | 321±8 ppm | 0.21%±0.01% |

*30 min at 60°C + 30 min at 75°C + 1 hour at 90°C; **2 hours at 90°C

**Table 15.2.3.7. Residual solvent concentration of ibuprofen agglomerates submitted to different drying conditions (Data from reference[3])**

| | Ethanol | Water |
|---|---|---|
| 2 hours at 40°C | 42±3 ppm | 0.76%±0.12% |
| 2 hours at 60°C | 21±4 ppm | 0.44%±0.06% |

As far as meprobamate spherical crystals are concerned, no significant differences are to be observed between drastic and progressive drying. No crusting phenomenon appears on meprobamate spherical crystals due to the loose tangling up of crystals, and so the solvent may escape easily between them.

The residual ethanol content of ibuprofen agglomerates is very low because of the open texture of agglomerates. Moreover, the crystallization phenomenon was relatively slow, enabling the solvent to escape from crystals in formation. The higher the temperature, the lower the residual ethanol content.

Both these crystals have a very porous texture. It seems that progressive drying is not essential as far as the polycrystalline particles are concerned.

If we consider the official limits[1,6] reported on Table 15.2.3.5 for residual solvent contents, we can note that the concentration of chloroform in meprobamate spherical crystals is much higher than the limit allowed in any drying conditions. Due to its inherent toxicity, this solvent should be avoided in the recrystallization process of meprobamate. The solvent

content obtained for ethanol in ibuprofen agglomerates is very low, in all drying conditions; in all cases it is considerably lower than the tolerated limits.

#### *15.2.3.2.3 Third example: polycrystalline particles of paracetamol*

Preparation of polycrystalline particles

Paracetamol agglomerates were prepared by the spontaneous precipitation of paracetamol into a mixture containing hexane, tetrahydrofuran and dichloromethane[9] under stirring. All the crystals obtained were filtered under vacuum.

Particle drying conditions

The melting point of paracetamol being 169°C and the transition temperature being 156°C, different drying conditions were tested (Table 15.2.3.2):

- Drastic flash drying: 156°C for either 10 or 30 minutes,
- Drastic drying: 100°C for 2 hours,
- Progressive drying: 60°C for 30 minutes, then 80°C for 30 minutes, and finally, 100°C for 1 hour.

After drastic drying at 100°C and progressive drying, a test with complementary drying at 156°C was carried out for 10 minutes.

All the recovered crystals were packaged in glass flasks before gas phase chromatography and other analysis.

Morphological aspect of particles

Several types of texture and morphology are to be observed in polycrystalline particles, according to solvent proportions. The most interesting and particular example are spherical polycrystalline particles which have a radial texture (spherolites) and appear as urchin-like particles (Table 15.2.3.3). SEM reveals the very typical surface crystallization of the agglomerates (Figure 15.2.3.9). They are made up of parallelepipedal flat crystals arranged perpendicularly to a central nucleus and they are relatively strong.

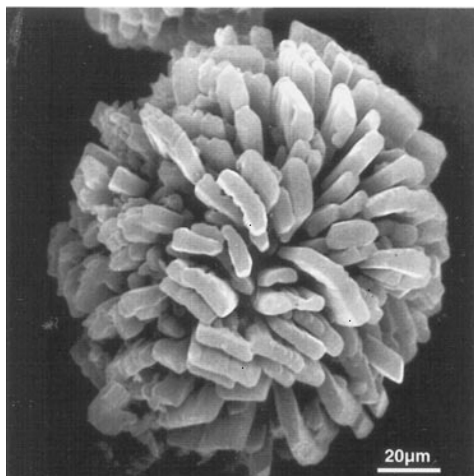


Figure 15.2.3.9. SEM photograph of paracetamol agglomerates (Photograph from reference[3]).

The implantation of peripherical crystallites is perpendicular to the surface. As it has been clearly demonstrated by Ettabia[10] a nucleus is formed first and then, in a second step, microcrystallites grow on it.

Residual solvent determination

For organic solvents, gas phase chromatography was performed on a Shimadzu GC-14B chromatograph fitted with a Flame Ionization Detector and a CR-6A Shimadzu integrator.

The packed column was Porapack super Q (Alltech, France), mesh range 80/100, length 1.80 m, internal diameter 2.16 mm. Carrier gas: anhydrous nitrogen. Injector temperature: 200°C. Detector temperature: 220°C. The chromatographic conditions were: For dichloromethane, tetrahydrofuran and hexane in paracetamol crystals: isotherm at 150°C, injection: 5 μl, RT: methanol 0.8 min, dichloromethane 3 min, tetrahydrofuran 7.8 min, hexane 11.8 min.

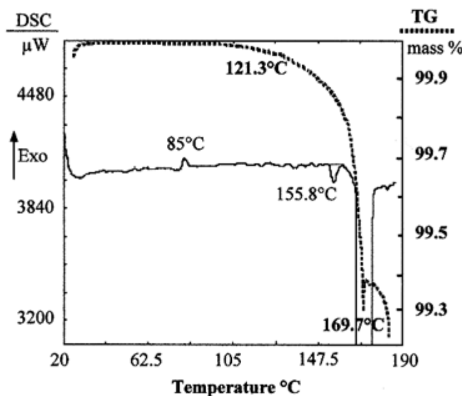


Figure 15.2.3.10. DSC and thermogravimetry curves of paracetamol agglomerates.

The residual solvent concentrations of the polycrystalline particles are reported in Table 15.2.3.8.

The wide-open texture of paracetamol spherolites hinders the crusting phenomenon; it is therefore normal that there should be no difference between the effectiveness of the two types of drying: progressive and drastic (at 100°C for two hours). However, a surprisingly good result is observed when the drying temperature is 156°C whereas a prolonged time at 100°C does not improve the solvent escape in spite of the low boiling points of solvents, all much lower than 100°C (dichloromethane: 39.5°C; tetrahydrofuran: 66°C; hexane: 69°C). Using a DSC method, it can be observed that a slight non constant exotherm at 85°C followed by a small constant endotherm at nearly 156°C occurs before the melting endotherm at 169°C (Figure 15.2.3.10). In fact, the paracetamol obtained by the crystallization process described is mainly the usual monoclinic form. However, as has been demonstrated by Ettabia[10] the formation of a certain amount of the amorphous form, causes the slight exotherm. The amorphous form recrystallized at 85°C into the orthorhombic metastable form, which transits into monoclinic form at 156°C, causing the small endotherm observed at nearly 156°C.

The thermogravimetric analysis shows that no solvation occurs during crystallization. Furthermore, the sudden solvent escape is not specific to one definite solvent, whereas all the contained solvent amounts dramatically decrease at 156°C. The loss of mass is high (about 0.7%) when the substance is about to melt (Figure 15.2.3.10).

**Table 15.2.3.8. Residual solvent concentration of paracetamol agglomerates submitted to different drying conditions (Data from reference[3])**

| | Dichloromethane | Tetrahydrofuran | Hexane |
|---|---|---|---|
| "Flash drying" 156°C | | | |
| for 10 min | < 200 ppm | 1095±5 ppm | 167±1 ppm |
| for 30 min | < 200 ppm | 630±24 ppm | 88±4 ppm |
| PD* | 315±3 ppm | 2066±1 ppm | 305±3 ppm |
| PD*+ “flash” (10 min at 156°C) | < 200 ppm | 883±10 ppm | 145±5 ppm |
| DD ** | 309±3 ppm | 2014±30 ppm | 313±4 ppm |
| DD**+"flash" (10 min at 156°C) | < 200 ppm | 873±2 ppm | 140±1 ppm |

*PD: Progressive drying (30 min at 60°C + 30 min at 80°C + 1 h at 100°C), **DD: Drastic drying (2 h at 100°C)

Paracetamol, containing three different solvents, exhibits low dichloromethane content (200 to 320 ppm). The referencing limits remain higher than the experimental results when these particles are submitted to different drying conditions (Table 15.2.3.5). The same observation is valid for tetrahydrofuran for which the allowed upper limit is 5000 ppm. But the residue in hexane is sometimes above the regulatory threshold. The only way to be definitely below this limit is to heat paracetamol to 156°C; this confirms the advantage of flash drying this substance. As previously described, this temperature is critical for paracetamol recrystallization in the solvents used.

This study enables us to underline some considerations about the drying conditions of crystals. It is important to note that not only should the usual parameters be taken into account, such as the melting point of the substances and the boiling point of the solvents, but also the texture of the particles and the thermodynamic events that can occur when the substance is heated.

The kinetic of temperature increase must be chosen with respect to crystalline particle texture to obtain optimal drying conditions for lowest RS content. Progressive drying can give better solvent elimination when the texture is very finely sintered to avoid the crusting effect, which hinders any ulterior solvent escape.[2]

When a solvate is formed, the drying temperature must be either equal to or higher than the desolvation temperature.

Lastly, the knowledge of the existence of solid-solid transitions could be in this respect of great interest. Thermogravimetry and DSC analysis are, among others, very efficient tools to assess these phenomena.

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