LANL Ref 1/1/1989

$50.00

(Section G - Liquid Scintillation Cocktails - Components of - LANL Reference)

LIQUID SCINTILLATION ANALYSIS

Science and Technology

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9489

Rev. G
Printed in U.S.A.
Publication No. 169-3052
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SAMPLE PREPARATION

INTRODUCTION

Probably one of the most important steps in the scintillation counting process is the sample preparation procedure. Most biologically radiolabeled samples, such as a piece of a plant (leaf, stem, root), a culture of cells, or an aqueous sample, cannot be placed in a vial and quantitated directly. The sample must be prepared so that the liquid scintillation analyzer is quantitating a homogeneous mixture of sample and scintillation solution. Therefore, it is extremely important to understand the different methods of sample preparation. This chapter and the next will cover the various methods of sample preparation; including aqueous samples, carbon trapping methods, proteinaceous tissue solubilizers, and sample oxidation (automatic and manual).

COMPONENTS OF A LIQUID SCINTILLATION SOLUTION

In order to understand what components are present in a liquid scintillation solution, it is necessary to understand the liquid scintillation process. This is shown in Figure 6-1. The process involves the transfer of kinetic energy from the beta particle (β^-) emitted by the radionuclide to solvent molecules. These excited solvent molecules then transfer their energy to scintillator molecules. When these molecules become excited, they return to their stable energy state by emitting photons with a certain intensity. The intensity of the light is directly proportional to the energy of the emitted beta particle.

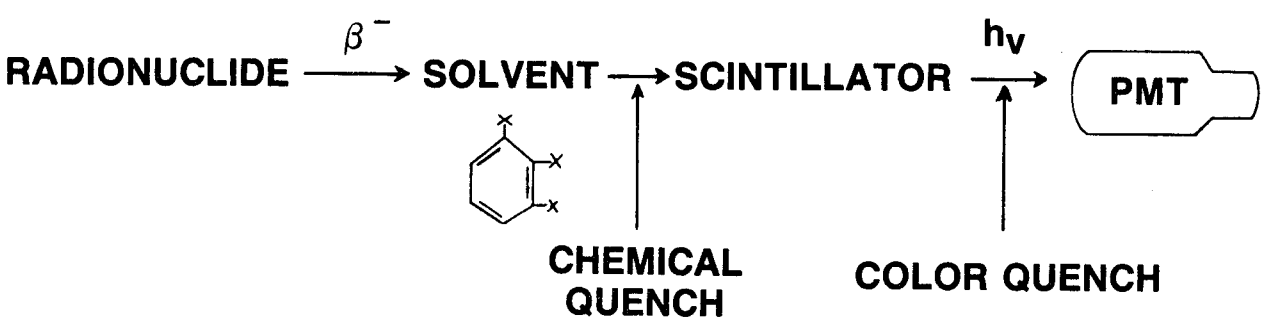


Figure 6-1. Basic Scintillation Process.

A classical aqueous-accepting scintillation solution is composed of several components: solvent, primary and secondary scintillators, and surfactant(s). Each of these components will be further explained:

1. Solvent - a chemical which converts the kinetic energy (β^-) of radiations into excitation energy and 'transfers' this energy to a scintillator molecule.
2. Primary scintillator (solute) - a chemical which converts excitation energy into photons.
3. Secondary scintillator (solute) - a chemical which shifts the light wavelength of emitted photons into an optimal wavelength response range of the photomultiplier tubes.
4. Surfactant (emulsifier) - a chemical which forms a stable, homogeneous emulsion through the formation of micelles with aqueous samples and organic solvents.

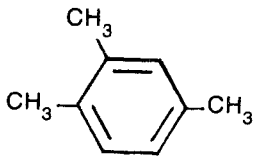
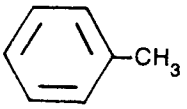
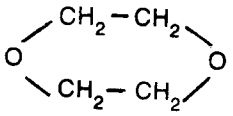
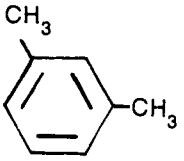
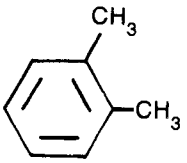
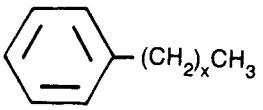
Solvent

The solvent acts as both a vehicle for dissolving the sample and scintillator, and the location of the initial energy transfer from the nuclear decay products to the solvent. The solvent must be able to transfer the energy without significant loss. The type of solvent molecules which are best able to do this are molecules which have nonbonding pi (π) electrons which can easily be excited to higher energies without the loss of energy. Typical molecules which display this π electron structure are aromatic molecules. In addition to the ability to transfer energy efficiently, the solvent must have the following characteristics:

1. It must readily dissolve the radioactive analyte and the scintillator molecules.
2. It must have a low content of natural ^{14}C.
3. It must have a high transparency to the photons of light emitted by the scintillator molecule.

A list of the most common solvents and their structure is shown in Table 6-1. Two characteristics of these solvents are worth investigating further: Relative Pulse Height (RPH) and flash point. The RPH is the pulse height measured for a radionuclide in toluene compared to an unknown solvent with the RPH for toluene referenced as 100. The flash point is defined as the lowest temperature at which the vapor of a combustible liquid can be made to ignite momentarily in air.

Table 6-1. Common Scintillation Solution Solvents.

Solvent	Chemical Structure	Relative Pulse Height	Flash Point °C
1,2,4 -Trimethylbenzene (pseudocumene)		112	50
p-Xylene		110	30
Toluene		100	5
Benzene		85	-11
1,4 -Dioxane		65	12
m-Xylene		107	25
o-Xylene		98	29
Alkylbenzene (high flash point)		91	150

The relative pulse height (RPH) is important because if this number falls below 100, the energy transfer and resultant output of photons is reduced. Thus, the higher the RPH, the better the solvent is for scintillation solution preparation. The flash point is also important. If the flash point is very low, the sample can easily be ignited and should be considered a potential fire hazard. If the solvent has a low flash point, it is normally more toxic (possibly even carcinogenic). Now let us examine each of these solvents individually.

One of the earliest solvents to be used was benzene but, because of its high toxicity, extremely low flashpoint, and the inability to quantitate aqueous samples in this solution, its use quickly became replaced with toluene and dioxane. Today, a special application for benzene is its use in low activity carbon dating. It is used in conjunction with the benzene synthesizer (converts sample completely to benzene for quantitation). By using this technique, large sample volumes can be incorporated into the scintillation solution.

Next, the solvent dioxane was used for counting aqueous samples using Bray's solution cocktail. However, dioxane-based cocktails exhibit many problems. First, they were not useful for aqueous solutions containing proteins, since dioxane will precipitate proteins. Second, sample types are limited because many materials will cause the naphthalene used in the Bray's cocktail to precipitate. Third, the dioxane is subject to auto-oxidation which produces peroxides (strong quenching and chemiluminescence production). These problems, together with a low counting efficiency, a low flash point and high toxicity (suspicious of being carcinogenic), have resulted in the mainly discontinued use of dioxane for liquid scintillation counting.

Next toluene was the solvent of choice. Toluene was readily available in high purity and at moderate cost. Together with TRITON X-100® (a nonionic surfactant) it formed one of the first emulsifier (colloidal) cocktails for counting aqueous-type samples. Toluene has several disadvantages as a solvent. The flash point for toluene is lower than room temperature; it can represent a significant fire hazard if not handled properly. The solvent toluene has also been proven to be highly carcinogenic, especially in the liver.

Xylene, a frequently used solvent, gives high energy conversion efficiencies and high relative pulse heights. The more common practice for using xylene as a solvent is to use one of the pure xylenes (ortho, meta, or para) or to mix two of them in a known ratio. Para-xylene is not useful by itself in controlled temperature liquid scintillation counters because it will freeze at 12-13 ° C. Although the xylenes are classified as flammable liquids, their flash points are above normal room temperatures, so they represent less fire hazard than the previous solvents.

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Pseudocumene (1,2,4-trimethylbenzene) has become a popular solvent for newly developed ready-for-use scintillation cocktails. First, it offers the highest energy conversion efficiency of the solvents known. Second, it is classified only as a combustible liquid by U.S. Department of Transportation regulations and, therefore, has few restrictions on shipping and storage. Third, it can be used with plastic vials, because the diffusion rate of this solvent through the vial walls is lower compared to the solvents mentioned previously.

Due to the toxicity, waste disposal, shipping and storage problems associated with the low flash point solvents previously discussed, a high flash point solvent was developed. This high flash point solvent, a long chain alkylbenzene derivative, has a low toxicity level and a high flash point (~150 °C), with only a slightly lower RPH (91). In conjunction with new emulsifiers necessary for aqueous samples, this new solvent gives excellent performance. It is also environmentally benign and, in many locations, is drain disposable. This high flash point solvent is found in Packard's Opti-Fluor® scintillation cocktail.

Scintillator - Primary

The scintillator molecules accept energy from the excited solvent molecules. The scintillator molecule then becomes excited to a higher energy state. The excited scintillators will then relax and return to the ground state with the concomitant release of energy. This energy is released in the form of a photon (light flash) which can be quantitated by the liquid scintillation analyzer. Thus, the scintillator must effectively accept the energy from the solvent and produce photons. The transfer must be relatively quantitative with the energy of the beta particle being directly proportional to the intensity of the photons released by the scintillator.

Several different types of primary scintillator are available for liquid scintillation counting (Table 6-2). The most popular are PPO, PBD, Butyl-PBD, and BBOT.

Four characteristics of each of the primary scintillators are presented. The first is the optimum concentration used in the preparation of the scintillation solution. If a higher scintillator concentration is used, quenching may result from the presence of excess scintillator. The second characteristic is that of the peak fluorescence of the scintillator. This value should be between 300 and 425 nm. This is the wavelength response of the photomultipliers (PMTs) which are used to detect the photons produced by the scintillator. The spectrum of PPO relative to the sensitivity of the photomultiplier sensitivity is shown in Figure 6-2.

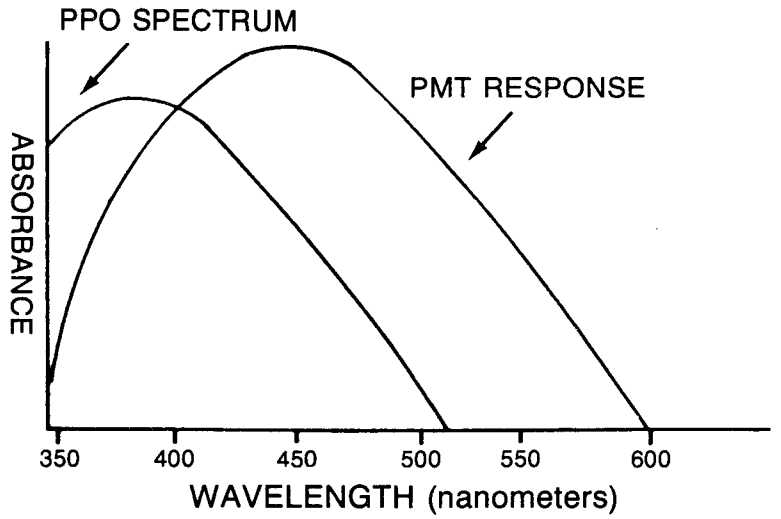
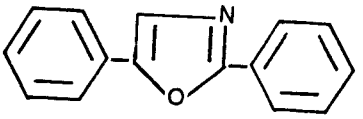
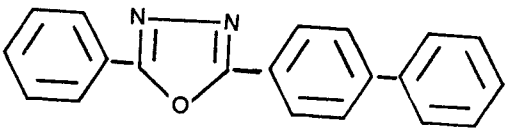
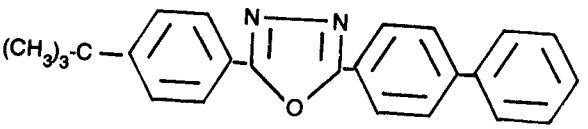
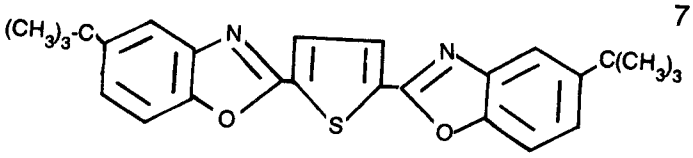


Figure 6-2. Spectrum of Primary Scintillator, PPO, and the Sensitivity (Response Range) of a Typical Photomultiplier.

The third charateristic is that of the decay time (*t*). This is very important, in that the photon peak must be very sharp. and not have tailing. this is most important at high count rates, when photons of various intensities can occur in very short periods of time. If the decay time is too long, then the photons created by beta particles at a high rate can be lost in the counting circuit. All modern scintillators typically have decay times of less than two nanoseconds (Table 6-2). The earlier scintillators, napthalene and diphenylanthracene, have decay times of 96 and 9.4 nanoseconds.

Table 6-2. Characteristics of Primary Scintillators.

Solute	Optimum solute concentration (g/liter)	Fluorescence maximum (nm)	Decay time t (nsec)	Quantum yield Φ
PPO 	5-7	375	1.4	0.83
PBD 	8-10	375	1.0	0.69
Butyl-PBD 	12	385	1.0	0.69
BBOT 	7	446	1.6	0.61

The fourth characteristic is the fluorescent yield, Φ. This is defined as the fraction of excited molecules which emit photons.

$$\Phi = \text{number of photons/number of excited molecules}$$

The closer the number is to 1.0, the better energy transfer and photon yield. In summary, each of the following primary scintillators have the following characteristics.

PPO (2, 5-diphenyl oxazole) is currently the most widely used primary scintillator. The reasons for the popularity of PPO are good scintillation efficiency (quantum yield) in moderate concentrations, good solubility in scintillation solvents, relatively low cost, and nonreactivity with most chemicals measured by liquid scintillation counting. For routine liquid scintillation applications, concentrations of five to six grams per liter will produce a cocktail with great resistance to chemical quenching. This enables higher counting efficiencies with lower concentrations of scintillator. In concentrations exceeding 10 grams per liter, PPO will act as a quenching agent of the scintillation process (self-quenching).

Butyl-PBD [2-(4-tert-Butylphenyl)-5-4 Biphenyl)-1,3,4 -Oxadiazole] is another efficient primary scintillator. It has three disadvantages. First, its cost is about double that of PPO. Second, the concentration required to achieve high efficiency is about twice that of PPO (recommended concentration: 12 grams/liter). Third, Butyl-PBD is chemically reactive. It will react with acids, bases, amines and other compounds. This reduces its effective concentration in the final cocktail. With basic samples Butyl-PBD produces a brownish color in the scintillation cocktail. This causes both chemical and color quenching in the sample.

BBOT [2,5-bis-2-(tert-Butylbenzoxazolyl)-Thiophene] is a primary scintillator which emits light (446 nm) mostly in the visible region. This property makes it less affected by optical quenching in the ultraviolet region. It has several disadvantages. First, it is only about 80% as efficient as PPO in converting excitation energy into light (quantum yield). Second, BBOT costs about twice as much as PPO and it is used in higher concentrations (recommended concentration: 7 grams/liter). Third, BBOT is chemically reactive. BBOT can react with acids producing a yellow to green color.

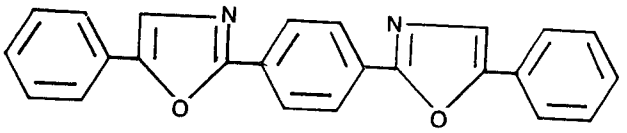
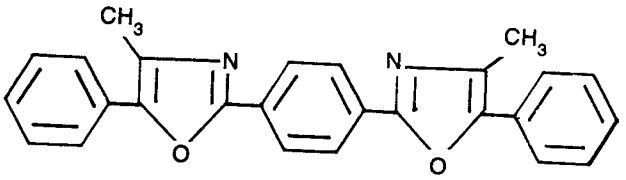
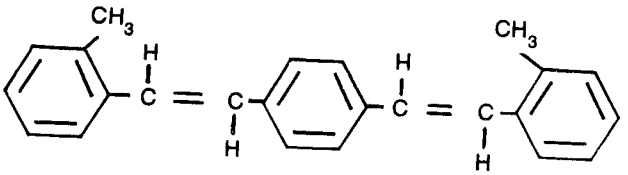
Scintillators - Secondary

Secondary scintillators (the third component in a liquid scintillation solution) were used in the early days of liquid scintillation counting in order to shift the wavelength of the photons emitted from 370 nm to 420 nm. The major reason for this was that the spectral response of the early photomultipliers was near 400 to 420 nm. Thus, the secondary scintillator acted as a wavelength shifter to change the wavelength of the photons emitted in the scintillation process. The exact mechanism of the second scintillator was not clearly understood. It was clear, however, that with the secondary scintillator present, the photons produced by the scintillation solution came only from the secondary scintillator. This was providing that the secondary scintillator concentration was sufficient to give 100% energy transfer from the primary to the secondary scintillator. Thus, a direct energy transfer from the primary to the secondary scintillator was necessary.

Today, a secondary scintillator is not absolutely necessary to shift the emitted wavelength because the modern photomultipliers are sensitive to the wavelength of the primary scintillator. The primary use today for secondary scintillators occurs when a large amount of color quenched sample is placed in the scintillation solution. The secondary scintillator provides a method to more effectively transmit the energy from the beta particle to produce light flashes which are directly proportional to the energy of the beta particle.

Three secondary scintillators are commonly used in preparing liquid scintillation solution: bis-MSB, POPOP, and dimethyl POPOP. The structures, wavelength average, and optimal concentration are given in Table 6-3.

Table 6-3. Properties of Secondary Scintillators.

COMPOUND	ABBREVIATION	CONCENTRATION (g/liter)	FLUORESCENCE MAXIMUM WAVELENGTH (nm)
	POPOP	0.05-0.2	415
	M_2-POPOP	0.1-0.5	427
	bis-MSB	1.5	425

The best general purpose secondary scintillator is bis-MSB [p-bis(o-Methylstyryl)-Benzene]. It is used as the secondary scintillator in most ready-to-use scintillation cocktails. Bis-MSB is readily soluble in solvents for liquid scintillation counting. It has a fast rate of dissolution. This makes it an extremely convenient secondary scintillator for laboratory-prepared cocktails. In high concentrations, it can function as a primary scintillator, supplementing the PPO concentration to increase resistance to quenching, and does not react chemically with most liquid scintillation samples (recommended concentration: 1.5 grams/liter).

POPOP [1,4-bis-[2-(5-Phenyloxazolyl)]-Benzene] was one of the earliest secondary scintillators used. It is still the most widely used in laboratory-made cocktails. It has low solubility, which limits the amount which can be incorporated into a scintillation cocktail. Its rate of dissolution is slow, thus resulting in long mixing times. POPOP is chemically nonreactive with most chemicals used in liquid scintillation counting (recommended concentration: 0.1 grams/liter).

Dimethyl-POPOP [1,4-bis-[2-(4-Methyl-5-Phenyloxazolyl)]-Benzene] is a derivative of POPOP which has higher solubility and faster dissolution rates in scintillation solvents. Dimethyl-POPOP is chemically reactive. It can react with acids to produce a yellow to greenish color (recommended concentration: 0.2 grams/liter).

Surfactants (Emulsifiers)

The fourth component of a liquid scintillation solution which accepts aqueous samples is a surfactant or emulsifier. This is probably the most important component of the aqueous-accepting scintillation solution because it provides the method for making a homogeneous solution between the aqueous sample and the organic scintillation solution. These emulsifiers hold the sample in intimate contact with the solvent by the formation of micelles. These surfactants are normally of two types: ionic and nonionic. Examples of each type are shown in Figure 6-3.

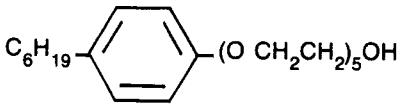
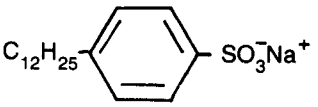
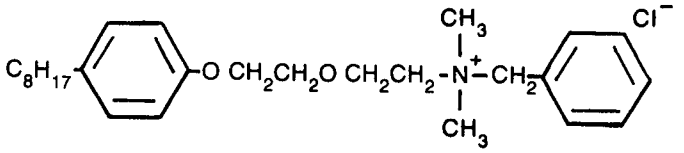
Name	Structure	Type
Triton N57		Nonionic
Dodecylbenzene sodium sulfonate		Anionic
Hyamine 166		Cationic

Figure 6-3. Surfactants Used in Scintillation Solution.

The nonionic surfactants are normally used to form micelles with water and certain salt solutions. The ionic surfactants have sample capacity for certain types of salt solutions.

The use of a nonionic emulsifier in a scintillation solution may result in up to three sample/scintillation solution types. The first is a clear homogeneous single phase which is liquid in nature. This is normally present for small sample loads. The next phase is a heterogeneous, two-phase solution, which cannot be counted effectively in a liquid scintillation analyzer. The two phases are a water phase and an organic phase. The third phase is a gel phase in which the sample and the scintillation solution form a clear gel. This is an effective solution for liquid scintillation counting. A typical phase diagram for a scintillation solution with various amounts of sample (sample load) is illustrated in Figure 6-4..

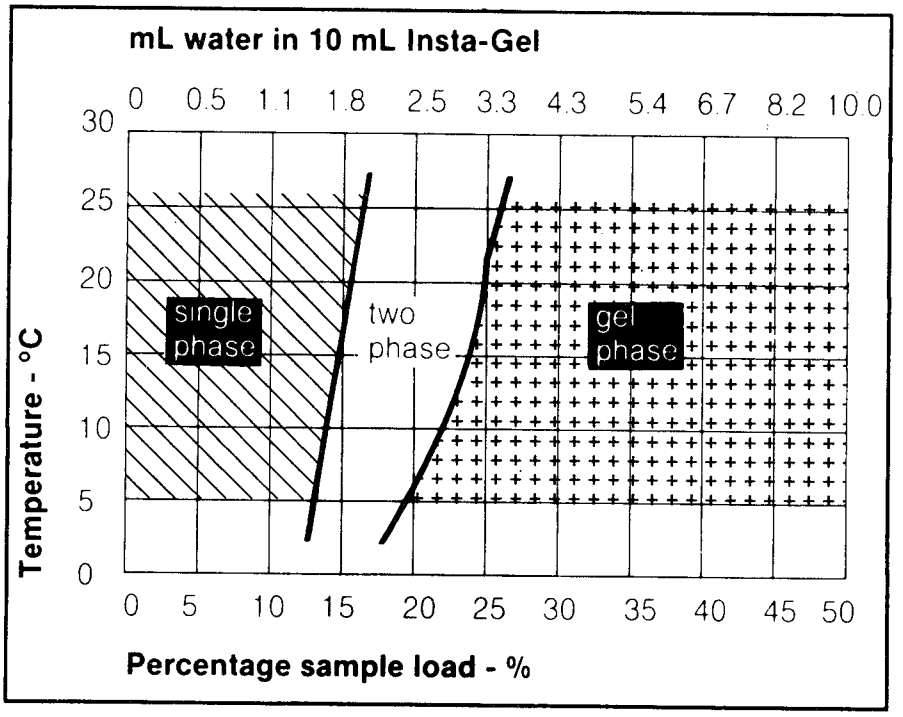


Figure 6-4. Phase Diagram for Insta-Gel Scintillation Cocktail.

In order to effectively analyze aqueous samples, it is important to use a phase diagram to determine whether the sample is being counted under homogeneous conditions necessary for liquid scintillation analysis.

SPECIAL APPLICATIONS USING SCINTILLATION SOLUTIONS

With the procedure described in the previous section, aqueous samples can be counted accurately if a single fluid phase or gel is formed, but many samples are not simple aqueous samples. These include:

1. Counting radioactivity from paper chromatograms.
2. Counting gamma or x-rays in a liquid scintillation analyzer.
3. Counting trapped ^{14}C-labeled carbon dioxide.
4. Counting tissue samples.
5. Counting samples on glass fiber filters.
6. Counting samples on membrane filters.
7. Counting TLC plate scrapings.
8. Counting slices obtained from gel electrophoresis.
9. Counting blood samples.
10. Counting insoluble particulates.

The major problem with most of the ten applications listed above is that the sample is not homogenous in nature. How does this affect the counting efficiency of the sample? This is illustrated in the following manner (Figure 6-5).

When a radionuclide decays it can emit a beta particle in any direction. If the radiolabel is dissolved in the scintillation cocktail, the radionuclide is completely surrounded by the detector and 4 π counting geometry is attained. (Solid angles are measured in steradians, 4 π steradians is the solid angle of a sphere). If the radioactive compound has precipitated, or is adsorbed to the vial wall or on a solid support (filter paper, etc.), the counting geometry is reduced to something less than 4 π. For adsorption and precipitation, the geometry is reduced to essentially 2 π, resulting in a loss of half of the detectable counts of the radionuclide. For higher energy nuclides, some of the beta particles can penetrate the solid support and be quantitated. If the labeled compound is on the surface of a solid support, the counting geometry is approximately 2 π; if the compound has penetrated into the solid support, then counting geometry can be even less than 2 π. For samples on solid supports, geometry is to some degree energy-dependent. If the beta particles emitted have adequate energy to pass through the solid support and interact with the scintillation cocktail, the geometry will be greater than 2 π. For very energetic beta particles, such as from ^{32}P, the geometry may approach 4 π. Thus, it is important especially for tritium (low energy beta emitter) to count with 4 π geometry (a homogeneous solution). The following procedures are methods which enable good counting geometries for the applications described below.

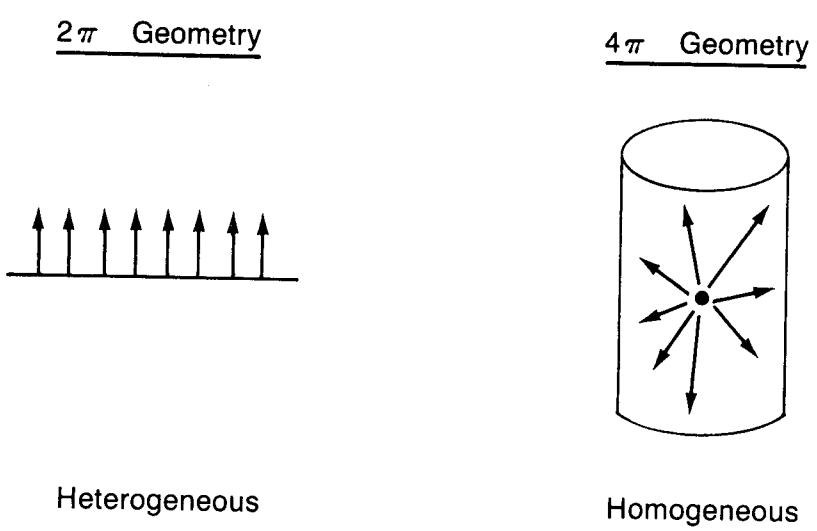


Figure 6-5. Comparison of Homogeneous and Heterogeneous Radioactive Samples.

1. Counting of Radioactivity from Paper Chromatography.

In order to quantitate samples from paper chromatography, it is important to elute the radioactivity from the paper chromatographic strip. In order to do this, it is important to assess the nature of the molecule on the paper chromatograph. Usually, paper chromatography is done with a particular solvent system. If the molecule moves rapidly on the paper chromatograph, then an easy method to elute the molecule is to use the same solvent used to develop the chromatograph. If the molecule moves very slowly on the paper chromatograph, then a solvent with the opposite polarity of the developing solvent should be used. The paper strips containing the molecule of interest are combined with the solvent and vortexed in the scintillation vial. If this solvent contains no heavy quenching agent such as chlorinated hydrocarbons, chloroform or carbon tetrachloride, scintillation solution can be added and the sample counted directly. In order to be sure that the radioactivity has been removed from the paper chromatogram strip, the strip can be removed and the sample only recounted. If the two counts are the same, then the radioactivity has been removed from the paper. The lower the energy of the radionuclide (^{3}H), the more important it is to elute the sample from the paper strip. If ^{32}P is in the sample, because of its higher (1700 KeV) energy, it can be quantitated without elution from the paper strip.

2. Counting Gamma and x-rays in a Liquid Scintillation Analyzer

Since many gamma and x-rays have very high energies compared to most beta particles, they can pass through the scintillation solution without transferring their energy to a solvent or a scintillator molecule. In order to quantitate these radionuclides, it is important to add a heavy metal compound such as tetrabutyl tin or tetraethyl lead to the scintillation cocktails. For higher energy gamma and x-rays, not even the addition of these heavy metals makes possible their quantitation.

3. Counting Trapped ^{14}C-Labeled Carbon Dioxide

There are two basic applications for the use of a carbon dioxide trapping agent: metabolism assessment and automatic sample oxidation. Two types of methods are used for trapping labeled carbon dioxide; inorganic and organic bases.

Solutions of inorganic bases have been used for a long time to trap carbon dioxide for liquid scintillation counting. Aqueous solutions of up to 1N sodium hydroxide or potassium hydroxide and methanolic solutions of 2N potassium hydroxide have been used routinely. The disadvantages of inorganic bases are three-fold. First, inorganic bases are strong quenching agents. Second they provide low trapping capacities. Third, they produce severe chemiluminescence. The major reasons for the use of inorganic bases is that their smell is preferred over the more pungent organic bases, and they are less expensive.

Organic bases, such as Hydroxide of Hyamine 10-X®, have been used to trap carbon dioxide for liquid scintillation counting. (Hydroxide of Hyamine 10-X is commercially available in a concentration of one normal in methanolic solution. It will trap about 0.5 millimole of carbon dioxide per mL. The proteinaceous tissue solubilizers are all organic bases and can be used to trap carbon dioxide.) These tissue solubilizers are toluene solutions with normalities of about 0.5N. These bases are fine for trapping small amounts of radiolabeled carbon dioxide.

For trapping large quantities of carbon dioxide, organic amines offer the best trapping capacities. Carbo-Sorb® is an amine which will absorb up to 5.8 millimoles of CO_2 per mL. Carbo-Sorb and the carbamate which results when CO_2 is trapped, are soluble in scintillation solutions containing toluene, xylene, or pseudocumene. These chemicals exhibit less quenching than inorganic bases. They will still produce chemiluminescence. In order to eliminate this chemiluminescence, one of three methods can be used.

First, the user can allow the chemiluminescence to decay to an acceptable level. Second, chemiluminescence can be corrected for by the instrument (see page 2-22). Third, chemiluminescence can be eliminated by selecting a cocktail which inhibits this chemiluminescence quickly to background levels (Hionic-Fluor™ - Packard).

The main applications of these trapping agents are summarized in Table 6-4.

Table 6-4. Carbon Dioxide Trapping Agents.

Trapping Agent	Properties	Uses
KOH) NaOH)solution	Low speed of trapping. Max. capacity (0.5-1.0 mmol/mL) depending on strength of solution.	Expired air. Low volumes of $^{14}CO_2$ to be trapped in 2-4 M solution.
Hydroxide of Hyamine® 10-X	Max. capacity of 0.5 mmol CO_2/mL.	Expired air. Reacts faster than KOH/NaOH
Soluene - 350	Lower maximum capacity than Hyamine 10-X. Reacts somewhat slower than Hyamine 10-X.	For trapping small volumes of $^{14}CO_2$
Methoxy-Ethyl-Amine	Reacts fast. Maximum capcity of 5-6 mmol CO_2/mL.	For trapping large volumes of $^{14}CO_2$ Not preferred for expired air experiments because of irritating smell. Used in Packard's sample oxidizer.

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4. Counting Tissue Samples

Since tissue and proteinaceous materials cannot be counted directly, they must be solubilized. This can be done either with a tissue solubilizer or with sample oxidation. The use of the sample oxidizer for this purpose will be discussed in detail in the next chapter of this manual. Now, the use of tissue solubilizers for digesting tissues, cells, and proteinaceous material are presented.

Packard Instrument Company offers the tissue solubilizer, Soluene-350. It is a toluene solution containing 0.5N of a specific quaternary ammonium hydroxide compound. Many samples can be solubilized at room temperature in less than two hours with 1.0 mL of solubilizer. Solubilization times can be shortened by heating the samples to 40 to 50 °C.

Soluene-350 solubilizer contains additional ingredients to improve water holding capacity and stability of the solubilizer. One mL of Soluene-350 solubilizer can hold up to 0.45 mL of water. This makes it an ideal solubilizer for tissue homogenates. Soluene-350 solubilizer has a rapid tissue-dissolving rate. Because of its greater stability, solubilizations can be performed at temperatures up to 50 °C, thus accelerating the solubilization process. Many samples can be solubilized at room temperature within a few hours. Soluene-350 exhibits the least chemical quenching of any of the known solubilizers.

In addition to tissue solubilizers to digest tissues, early work was done using a methanolic solution of hydroxide of Hyamine 10-X. Hydroxide of Hyamine 10-X is chemically identified as p-(diisobutylcresoxyethoxyethyl) dimethyl benzyl ammonium hydroxide. Its strength as a tissue solubilizer is relatively low at room temperature compared to the Soluene tissue solubilizer. It also exhibits a large amount of chemical quenching due to its methanolic base. On the other hand, this methanolic base will reduce the chemiluminescence resulting from this basic ammonium compound.

Special care should be taken when using tissue solubilizers. They are designed to solubilize proteins and tissues; therefore, they will cause severe burns when they come in contact with living tissue. Solubilizations should be performed in tightly capped vials, because air will react with the solubilizers, causing color formation.

5. Counting Samples on Glass Fiber Filters

The use of glass fiber filters is useful in applications using a cell harvester (receptor binding, cell propagation studies, disease diagnostics, and release studies) and DNA/RNA, DNA/DNA hybridization studies. In order to quantitate samples on glass fiber filters, it is necessary to elute the radioactive material from the filter. In order to do this, it is necessary to know the chemical nature of the radiolabeled material. If the sample is completely soluble in the scintillation fluid, then the sample can be quantitated directly. Examples of soluble samples are some drugs, lipids, and fatty acids. If the sample is not soluble, then place the wet filter in a glass scintillation vial. Add 10 mL of Insta-Fluor/Soluene -350 (9:1) and quantitate in a liquid scintillation counter. The Insta-Fluor is an organic based scintillation solution, and the Soluene -350 is a powerful tissue solubilizer. This procedure can also be used efficiently on cellulose acetate, Teflon® , Duralon® , and Fluoropore® filters. In addition, a specialized scintillation solution, Filter-Count, can be used for membrane filters of cellulose nitrate. A detailed description of the procedure will be described in the next section on counting membrane filters.

6. Counting Samples on Membrane Filters

In many biochemical applications, special membrane filters are used to quantitate various radiolabeled products. These membrane filters include cellulose nitrate, mixed cellulose esters, and P.V.C. filters. In order to effectively quantitate radioactivity on these membranes, the procedure for glass fiber filters could be used, but for some membranes the Soluene -350 turns the solution yellow and makes accurate quantitation difficult. An alternative to this procedure is the use of Filter-Count specialized scintillation solution, which does not cause color in solution. This scintillation solution can dissolve many membrane filters and also solubilize the radioactive samples. The wet or dry membrane filter is placed in a scintillation vial and 4 to 10 mL of Filter-Count is added. After 15 minutes, the sample is shaken and quantitated in the liquid scintillation counter.

7. Counting Thin Layer Chromatographs (TLC)

Two methods of TLC can be performed in order to separate various radiolabeled components: glass and plastic TLC. For glass backed TLC plates, the sample is first applied, the chromatography performed, and the glass TLC allowed to dry. After this, the lanes containing the radiolabeled samples, are divided into 0.5 to 1.0 mm slices with a pencil. A razor blade or scoring device is then used to remove each 0.5 to 1.0 mm section from the glass backing. The scraped area can then be removed and placed into a scintillation vial. This process is very tedious and time-consuming. If a plastic backed TLC plate is used, each section 0.5 to 1.0 mm can be cut out and placed directly in a scintillation vial. The next step is to dissolve the radiolabeled material from the thin layer material. This can be done by assessing the nature of the radiolabeled material and determining what solvent is necessary to remove the radioactivity from the TLC plate. Once this is done, then 1 to 2 mL of the solvent

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is added to the scintillation vial and allowed to set for 10 to 15 minutes. At this point, an aqueous scintillation solution, Hionic-Fluor or Insta-Fluor, can be added (10-15 mL) to each sample. The radiolabeled material is then quantitiated in a liquid scintillation counter.

Another method of counting TLC scraping is suspension counting for ^{14}C or higher energy isotopes. Excellent results can be obtained. For complete details see next page.

8. Counting Polyacrylamide Gel Slices Obtained from Electrophoresis Samples

Many applications involve the use of polyacrylamide gel electrophoresis (PAGE) to separate DNA, RNA, proteins, peptides, and other molecules. In order to quantitate radioactivity from the PAGE separated sample, four different procedures can be used:

1. Solubilizing
2. Macerating
3. H_2O_2
4. $H_2O_2/HClO_4$

First, the procedure using a tissue solubilizer will be described. A gel slice (1 to 3 mm) is placed in the bottom of a glass scintillation vial. A solution containing 9 mL of Insta-Fluor and 1 mL of Soluene - 350 is added to the vial and it is closed tightly with a polyethylene lined cap. The vial is placed in a waterbath at 50 °C for 2 to 4 hours or overnight at room temperature. The gel will appear swollen and transparent. The sample is then counted in a liquid scintillation analyzer after temperature and chemiluminescence stabilization.

Second, the gel slice can be macerated (broken up with a glass rod) in 5 mL of water and incubated at 50 °C for four hours. After the incubation 10 mL of Insta-Gel is added to the sample before quantitation.

Third, the gel slice is incubated with 2 mL of 30% H_2O_2 for 5 hours at 50 °C. After the incubation 12 mL of Hionic-Fluor is added to the sample.

Fourth, the gel slice is incubated in 1 mL of 30% H_2O_2 and 1 mL $HClO_4$ for 2 hours at 80 °C. After the incubation 15 mL of Pico-Fluor 15 is added to the sample before quantitation.

In summary, the Insta-Fluor/Soluene method gives high counting efficiency, high recoveries, fast results, and is easy to perform. This is the best of the four procedures. The wet oxidation method (three and four), gives high recoveries but reduces counting efficiency. Also, H_2O_2 may react and alter some of the radiolabeled material, and causing loss of radioactivity in gaseous forms and under some circumstances explosion hazard may result in the glass scintillation vial. This treatment results in lower counting efficiencies. Macerating and elution with water give good results for water soluble radioactive compounds, but are counted with lower efficiencies.

9. Counting Blood Samples

Many experiments require quantitation of radiolabeled material in blood. These experiments are used to monitor clearance rate studies of new drugs and other applications. The procedure involves adding 0.1 to 0.4 mL of whole blood to a 1 mL mixture of Soluene-350/isopropanol, 1:1. This mixture is incubated at 40°C for 15 to 30 minutes. At the end of the incubation, 0.5 mL of 30% hydrogen peroxide is added slowly (dropwise) with constant stirring. This mixture is then allowed to set for 30 minutes at room temperature. At the end of this time, 10 mL of Hionic-Fluor are added to the sample. The sample is then allowed to equilibrate in the instrument for 30 minutes before counting, in order to stabilize temperature and chemiluminescence.

10. Counting Insoluble Particulates

Cab-O-Sil® , a thixotropic gel powder, can be added to a scintillator solution in concentrations of 3 to 4% (w/v) in a vial to form stable gels. Under mechanical agitation the mixture becomes a fluid, returning to a gel when agitation is stopped.

This preparation is used to suspend insoluble particulates (e.g. radiolabeled barium carbonate samples) for counting. In lower concentrations (1 to 2%) Cab-O-Sil is used to provide a large surface area to minimize adsorption of samples to the vial wall.

The previous ten applications provide methods necessary to form a homogeneous counting solution from a heterogeneous sample. This homogeneity provides 4 π counting geometry and, consequently, accurate DPM determination of the hard-to-quantitate sample.

CHOOSING A SCINTILLATION COCKTAIL

Choosing the appropriate scintillation cocktail is very important in liquid scintillation analysis. The combination of the sample and the scintillation solution is a major determining factor of the quality data (DPM) in the counter. Several factors are important in the quality data:

1. Sample compatibility
2. Counting performance
3. Cost
4. Convenience
5. Safety and disposability

These factors are all interrelated, and each will be discussed separately.

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Sample Compatibility

The primary criterion for choosing a scintillation cocktail is sample compatibility (the compatibility between the scintillation cocktail and analyte or sample to be measured). One factor affecting sample compatibility is the solubility of the sample in the cocktail. The optimum counting performance is achieved when the sample is in solution with the scintillation cocktail. The radionuclide in the analyte is then in intimate contact with the primary solvent. If the analyte is in solution, the sample is in a homogeneous phase; if the analyte is not in solution, the sample is in a heterogeneous (multiphase) state. The term 'phase contact' is used to describe the degree of homogeneity of the sample; 'good phase contact' describes those samples which count as solutions. In a scintillation cocktail, the organic phase is the medium in which the energy conversion process occurs. Radionuclides not in the organic phase (described earlier - precipitated, adsorbed, or in a separate liquid phase) will yield a lower counting efficiency than the potential counting efficiency of the cocktail. Phase contact can be quantified using standards (samples of known activity). The counting efficiency (C.E.) is determined using a known activity of the labeled compound in the analyte or by using a radioactive compound that will distribute in the cocktail the same as the analyte (e.g., ^{3}H-water). The cocktail counting efficiency (Ec) is determined using a soluble standard in the organic phase of the cocktail (e.g., ^{3}H-toluene). The chemical and optical quenching should be the same in the two samples. The ratio of these efficiencies provides a quantitative measure of phase contact (P.C.).

$$\text{P.C.} = \frac{\text{C.E.}}{\text{Ec}}$$

The closer the value of P.C. is to 1.0, the better the phase contact. At a value of 1.0, the radionuclide is being counted as a true solution. Values <1.05 or $>.95$ give questionable results.

The amount of sample a scintillation cocktail will hold is its sample holding capacity, expressed as the sample load (SL). For liquid analytes, sample load is usually expressed as a percentage, and is the ratio of the volume of sample to the volume of the sample plus cocktail.

$$\%\ \text{Sample Load} = \frac{\text{mL sample}}{\text{mL sample} + \text{mL cocktail}} \times 100$$

The relationship between sample volume, cocktail volume and sample load is shown in Figure 6-6.

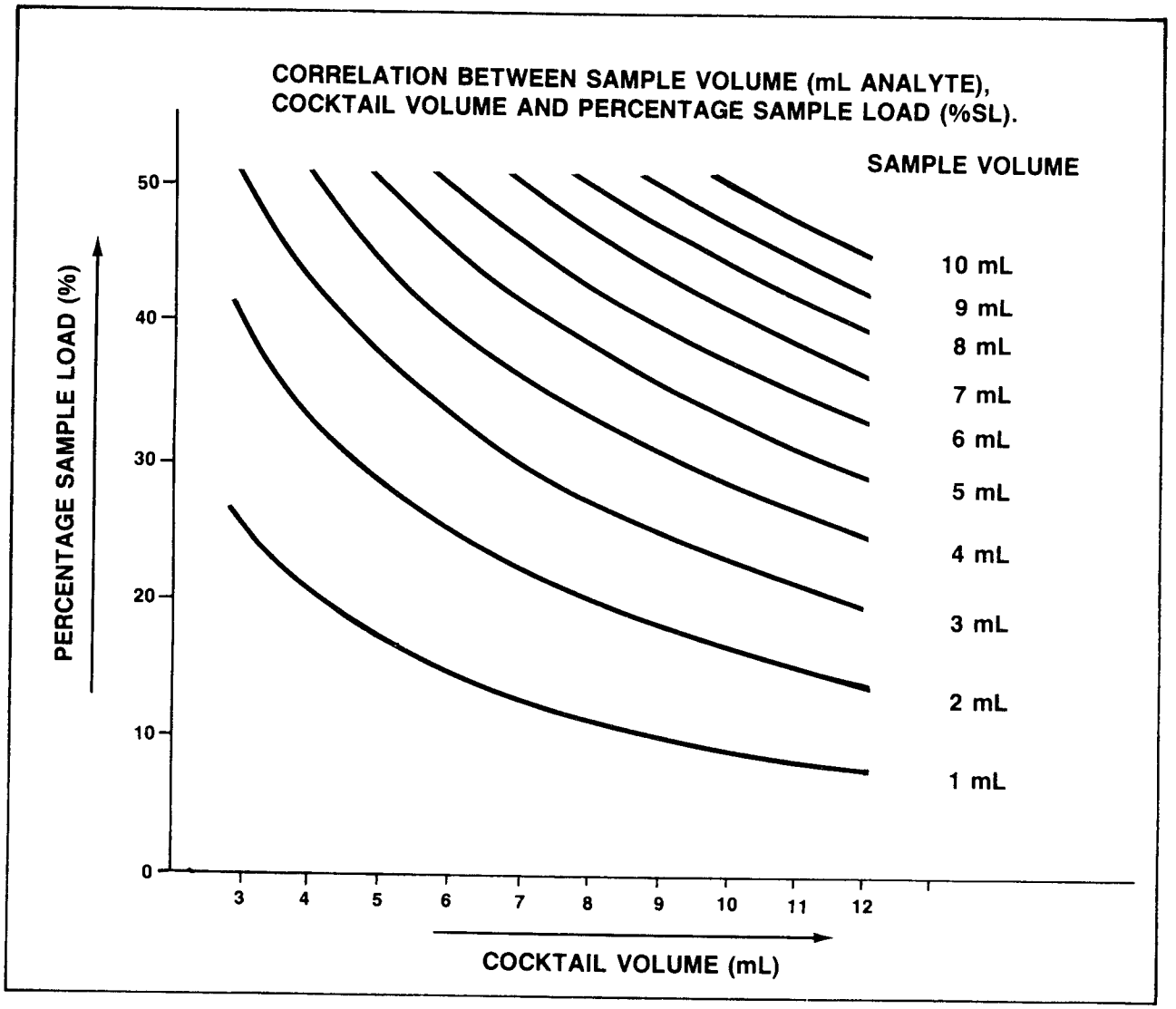

Figure 6-6 Correlation Between Sample Volume (mL Analyte), Cocktail Volume and Percentage Sample Load (%SL).

For aqueous types of samples, a hydrophilic scintillation cocktail is required. The sample holding capacity of these types of cocktails is determined by the emulsifier system and the solvent. Together they form a specific capacity for a certain analyte. This sample contains the labeled compound, but may also contain concentrations of other solutes (e.g., salt/buffer solutions). If these solutes exceed the maximum sample holding capacity of the cocktail, then precipitation, phase separation, or a milky appearance of the sample may occur, resulting in incorrect counting performances.

Visual inspection of the homogeneity of the sample may not always be adequate. In case of doubt, apply the phase-contact method for checking homogeneity, or use the sample homogeneity monitor of the liquid scintillation analyzer.

For nonaqueous organic solutions, lipophilic scintillation cocktails can be used, although these types of samples can be counted in the emulsifier-type cocktails (universal scintillation cocktails). The lipophilic cocktails will normally give higher counting efficiency and are less expensive.

Counting Performance

The second criterion which affects quality of the measured data is the counting performance of the scintillation cocktail. The better the counting performance of the cocktail, the shorter is the counting time required to achieve a desired statistical precision, or the greater the statistical precision which can be achieved in a given period of time. Also, depending on the type of experiment, less radioactivity can be used with the more efficient cocktail to achieve the same statistical precision.

Counting performance is related to both the efficiency of measuring the radioactivity in the analyte and the sample holding capacity of the cocktail. Cocktails do have correct counting efficiencies with a certain amount of analyte if the Phase-Contact value, with that specific amount of analyte, is equal to one (P.C. = 1.0).

To evaluate the counting performance of a particular cocktail, the Figure of Merit (FOM) is used. The most useful formula of the FOM for evaluation of counting performance is shown in the equation below (E = counting efficiency and V = sample load).

$$FOM \alpha E \times V$$

In this form, the FOM can be used to compare the counting performance of scintillation cocktails. The cocktail which has the largest FOM will give the best counting performance for that analyte. For any single cocktail, the sample load (V) which gives the largest FOM for that analyte will give the best counting performance. If the volume of analyte is limited, the cocktail which has the highest counting efficiency for that volume of the analyte will give the best counting performance.

To evaluate cocktails according to the FOM requires measurements of the counting efficiency at different sample loads of specific samples. For cocktails which are used for a variety of analytes, a more convenient criterion for comparative evaluation is the quench resistance of the scintillation cocktail. Cocktails which are most resistant to chemical or optical quenching will exhibit a small loss of counting efficiency with increased volumes of the quenching material. Quench resistance can be defined as the slope of the correlation curve for efficiency versus volume of the quenching agent. The lower the value of the slope of this curve, the greater is the quench resistance for the cocktail. Generally, the scintillation cocktail with the greatest quench resistance will give the best counting performance for any analyte over its range of sample holding capacity.

The quench resistance of different ready-to-use cocktails, is illustrated in Figure 6-7.

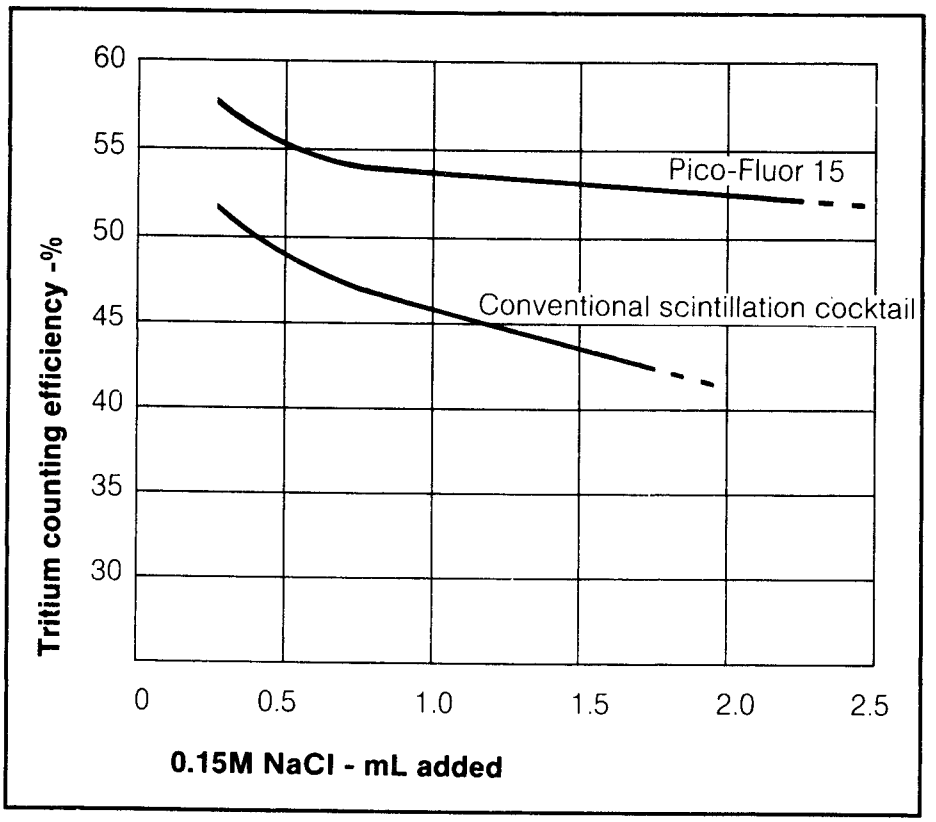

Figure 6-7. Quench Resistance of Packard Pico-Fluor™ 15 Versus Conventional LSC cocktails.

Cost

Cost may be an important consideration in choosing a scintillation cocktail. Economy can be achieved by using lower cost materials or by reducing the amount of materials used. Generally, the lower cost scintillation cocktails produce lower counting performance. Often, greater economies can be achieved without sacrificing counting performance by choosing a higher performance cocktail which could result in a reduction of the volume of scintillation solution. The volume reduction will depend upon the sample holding capacity of the high performance cocktail. For example, consider tritiated analytes of 1.0-2.0 mL diluted aqueous salt solutions counted in 10 mL of a Triton-X-100/Toluene solution with counting efficiencies of 42 to 37% respectively. In 5 mL of Pico-Fluor 40, these analyte volumes would count with efficiencies of 40 to 35%. Thus, by changing to Pico-Fluor 40, the volume of cocktail used can be reduced by 50% without sacrificing efficiency.

This volume reduction also results in a lower volume of radioactive waste. This, together with the use of small vials, results in a significant cost reduction per sample, even if Pico-Fluor 40 were to cost twice as much as a low price, low performance cocktail.

When comparing costs of scintillation cocktails, it is important to include all the costs involved. Commercially prepared cocktails generally cost more than the cost of the materials for similar laboratory-prepared cocktails. For laboratory prepared cocktails, there are a number of hidden costs which should be added to the material cost of the cocktail. The largest of these costs is the cost of preparation of the cocktail. This includes the labor to make the cocktail plus the expense of any special labware required for the preparation of the cocktail. The preparation cost of a laboratory-prepared cocktail usually includes a minimum of two hours of a technician's time per lot (typically four liters only).

An expense which applies to both commercial cocktails and laboratory-prepared cocktails is the cost of quality assurance. Each lot of cocktail should be tested for counting performance and sample compatibility, to ensure that correct and consistent results are obtained, if compared to previous batches.

This cost is usually higher on laboratory-prepared cocktails, since lot sizes are typically only one gallon. In laboratories which use large quantities of cocktails, lot sizes may be five gallons, which requires special labware to prepare the cocktail and requires more preparation time. An advantage of buying commercially prepared cocktails in large quantities is that the quality of the cocktail from a single lot will be assured and quality assurance time will be minimized. In addition, the manufacturers of commercial cocktails must meet quality assurance standards and will stand behind their product.

Another cost associated with laboratory-prepared cocktails is defective material or material being unsuitable for liquid scintillation counting. A cocktail that does not perform as required must either be reworked to improve performance, used as is, or scrapped. Reworking or scrapping a cocktail is expensive. Using the defective cocktail is perhaps the most expensive alternative, since this may lead to incorrect experimental results and may require repeating the entire experiment.

Except for the material cost, the expenses of a scintillation cocktail are hidden in the operating expenses for the laboratory. They are, nevertheless, real expenses, since time spent making the cocktail is time lost doing more productive projects. When all costs are included, commercial cocktails are usually more economical than comparable laboratory-prepared cocktails.

Convenience

Convenience is another important consideration in choosing the scintillation cocktails. Convenience usually translates into a time (cost) savings or improved quality of data. For example, the use of a single, universal cocktail is a convenience, and it provides improved quality of data, since there is no chance of using the wrong cocktail in an experiment. The users become familiar with its performance, its sample holding capacity and, if DPM is calculated, the confidence of efficiency determination applicable to all experiments. Costs can be reduced, since more of a common cocktail is used (purchasing in larger quantities usually reduces the unit cost on any item), less time is spent finding the appropriate cocktail, and experiments have to be repeated less frequently.

Commercial cocktails are convenient, since no time is required to prepare the cocktail. These cocktails are usually packaged in containers which are convenient to use. Cost savings have already been discussed for the commercially produced cocktails. These ready-for-use cocktails may also provide improved counting performance, since impurities which affect counting performance and sample holding capacity are removed during manufacture, enabling a comparison with previously obtained results.

Safety

An important aspect for choosing an LSC cocktail today is safety. Safety should always be a concern in the laboratory. With scintillation cocktails two aspects of safety are important: fire and health. Most solvents used in scintillation cocktails are flammable and also have toxic properties. Whenever scintillation cocktails are used or handled, procedures should be adopted to reduce or eliminate the hazards.

The hazard of fire is a major concern, since many materials in the laboratory will readily burn. The Department of Transportation has divided liquids which will burn into two categories for regulatory purposes: 1) flammable liquids, which have flash points below 100 °F (TCC) and 2) combustible liquids, with flash points of 100 °F or higher.

To evaluate the relative fire hazard of scintillation cocktails, it is necessary to understand what flash point means. The flash point of a liquid is the lowest temperature required to evolve an adequate vapor concentration so that the vapors can be ignited by an open flame near the surface of the liquid. The fire hazard is decreased for any liquid with a flash point above the laboratory's ambient temperature.

Table 6-5 lists the flash points for a number of commonly encountered liquids. Common solvents for scintillation cocktails are marked with an asterisk. As can be seen from this table, scintillation cocktails have flash points comparable to or higher than many other commonly used liquids. Special consideration should be given to the high flash point solvent used in Opti-Fluor scintillation cocktail.

Table 6-5. Flash Points of Some Common Liquids.

	°F	°C
High flash point solvent of Opti-Fluor* and Opti-Fluor O.	302	150
Pseudocumene*	118-122	48-50
Xylenes*	78-84	25-29
Isopropyl alcohol	72	22
p-Dioxane	54	12
Ethyl alcohol (absolute)	48	9
Toluene*	41	5
Benzene	12	-11
Gasoline (isooctane)	10	-12

Liquid scintillation cocktails with higher flash points generally offer the following advantages:

* Safer to use
* Fewer regulations for usage and storage.
* Fewer restrictions for transportation
* Lower vapor pressures, resulting in less vapor released in the instrument and working area.
* Less diffusion through plastic vials, resulting in a more constant counting volume, a lower "wall-effect," etc. Especially with the very high flash point solvents, as used in Opti-Fluor, no diffusion is observed; therefore, no 'wall effect' occurs.

The health hazard may be a more important safety consideration with scintillation cocktails. Excessive exposure to the solvent vapors may cause headaches, nausea and dizziness. Prolonged exposure may cause fainting. Table 6-6 provides data to compare the relative health hazard of some primary solvents used in liquid scintillation cocktails.

Table 6-6. Toxicity Values on Some Primary Solvents.

	Vapor pressure[1] at 25°C in mm. of Hg.	Equilibrium[2] Vapor Concentration at 25°C, ppm	Threshold Limit Value (TLV)[3] ppm	Toxicity Ratio[4]
High Flash-point solvent[5]	0.075			
Pseudocumene	2.2	2,900	25	116
p-Xylene	8.8	11,600	100	116
m-Xylene	8.2	10,800	100	108
o-Xylene	6.6	8,700	100	87
Toluene	28.2	37,100	100	371

[1]Calculated from data in the 'Handbook of Chemistry and Physics,' edition 19.
[2]Calculated for barometric pressure of 760 mm Hg.
[3]'1978 Registry of Toxic Effects of Chemical Substances,' published by the National Institute of Occupational Safety and Health.
[4]Toxicity ratio is the ratio of equilibrium concentration to TLV.
[5]For the high flash point solvent in Opti-Fluor, no TLV value is assigned.

The Threshold Limit Value (TLV) of a substance is defined as the maximum concentration of a substance in air to which most humans can be exposed for a period of time without exhibiting any toxic effects. Lower TLV values indicate greater risk of toxic effects. The lower the vapor pressure of a liquid, the lower is the rate of evaporation and the less is the exposure to vapors. These two factors must both be considered when evaluating the toxicity hazard of solvents. A toxicity ratio, the ratio of the equilibrium vapor concentration to the TLV, can be used. This provides a convenient means for evaluating the toxicity hazard for liquids. A low toxicity ratio indicates a lower toxic hazard.

The toxicity ratios for solvents used in liquid scintillation cocktails are all relatively high; the only way to ensure a low toxicity hazard with these solvents is to provide adequate ventillation in areas where the solvents are used and stored. Use of dispensing devices will also limit the amount of vapors which can escape into the air.

The high flash point solvent used in Packard Opti-Fluor cocktail exhibits the lowest toxicity when compared with commonly used solvents.

Volume of Cocktail Used

In selecting the volume of cocktail used, the same criteria apply as for choosing a cocktail. Traditionally, 10 to 15 mL of scintillation cocktail are used. With the advent of small vials, as little as 3 mL of scintillation cocktail can be used with good counting performance. For small volumes of cocktail, chemical quenching becomes more of a problem because quenching is dependent upon the analyte concentration.

Reducing the cocktail volume to one-half for a given volume of analyte nearly doubles the concentration of quenching agents in the sample. Low cost cocktails, either laboratory-made or commercially prepared, usually have low scintillator concentrations and are therefore more subject to quenching (they have less quench resistance).

For good counting performance, cocktails in high scintillator concentrations and an optimum emulsifier system (having a relatively high quench resistance together with the analyte), are needed.

The major advantage of converting to small vials is cost savings. Small vials cost less than conventional vials of the same material. Often the reduction in volume of scintillation cocktail will result in savings, even if a more expensive cocktail is required to achieve the desired counting performance. A major cost savings also comes from reduction of radioactive waste disposal.

The major limitation of using small vials is the volume of analyte which can be measured. In a conventional vial, analyte volumes up to 10 mL can be measured with good counting performance. For small vials, which have total capacities of 6 to 7 mL, the largest practical volume of analyte is 3 mL.

Summary

Choosing a cocktail for liquid scintillation counting is a very important part of the experiment design. For correct counting data, the cocktail must be compatible with the analytes to be counted. Deciding which cocktail to use is therefore a compromise between counting performance, cost, and convenience. Cost is not necessarily determined by the price of the scintillation cocktail alone. The volume of cocktail used, the cost of vials, disposal cost, and other costs associated with the cocktail would be included when evaluating cost per sample.

Safety is always an important concern in the laboratory. A scintillation cocktail with a flash point above the laboratory temperature is less of a fire hazard than a cocktail with a low flash point. Health hazards are the most significant hazards associated with scintillation cocktails. These hazards can be minimized by proper handling of the scintillation cocktails and good ventillation.

The use of the new generation of LSC cocktails, showing a much lower vapor pressure, will certainly improve safety in the work area.