

GETTING THE BEST FROM YOUR AGILENT LIGHT SCATTERING DETECTOR GUIDELINE

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1.0 SUBJECT

This document describes the recommended “housekeeping” steps necessary to get the best performance from your light scattering detectors and help prevent unnecessary service. It also gives a description of the cell cleaning procedure and an overview of the light scattering theory.

2.0 SCOPE

The following procedures are applicable to all Agilent 15/90 light scattering detectors including the Agilent PL-GPC 220 Dual Angle LSD, Agilent 1260 Infinity MDS Dual Angle LSD and Agilent PL-GPC 50 Dual Angle LSD systems.

3.0 COMMON PROBLEMS

1. **Solvent should be filtered** - Since light scattering is extremely sensitive to particles in the chromatography system it is essential that the solvents used are as clean as possible. It is recommended therefore that before introducing any solvent they are filtered down to at least 0.1µm and ideally 0.02µm. This includes flushing solvent and the solvent used to dissolve the sample, throwing away the first 20mls to pass through the filter. There is an excellent range of inorganic membranes sold under the Anotec brand that are ideal for this.

Aqueous eluent – Aqueous buffers contain more particulates than organic solvents and extra stringent filtering should be employed. *After* the salts have been added and dissolved filter through at least 0.1µm, three times through the same membrane.

2. **Columns should be flushed** – New columns are particularly prone to contain small fragments of packing material called fines that will wash off the column when first used. It therefore is necessary to flush the columns with the solvent you are to use to wash off these fines before connecting to a light scattering detector. Older columns too can release a high level of particulates especially when the solvent is exchanged in the column and more often when the flow rate is abruptly changed on the system. When setting up a new system or changing the solvent in a column set it is good practice to flush the columns directly to waste and pass

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through the column at least 10 column volumes of solvent before the columns are connected to the light scattering detector, at the analysis flow rate.

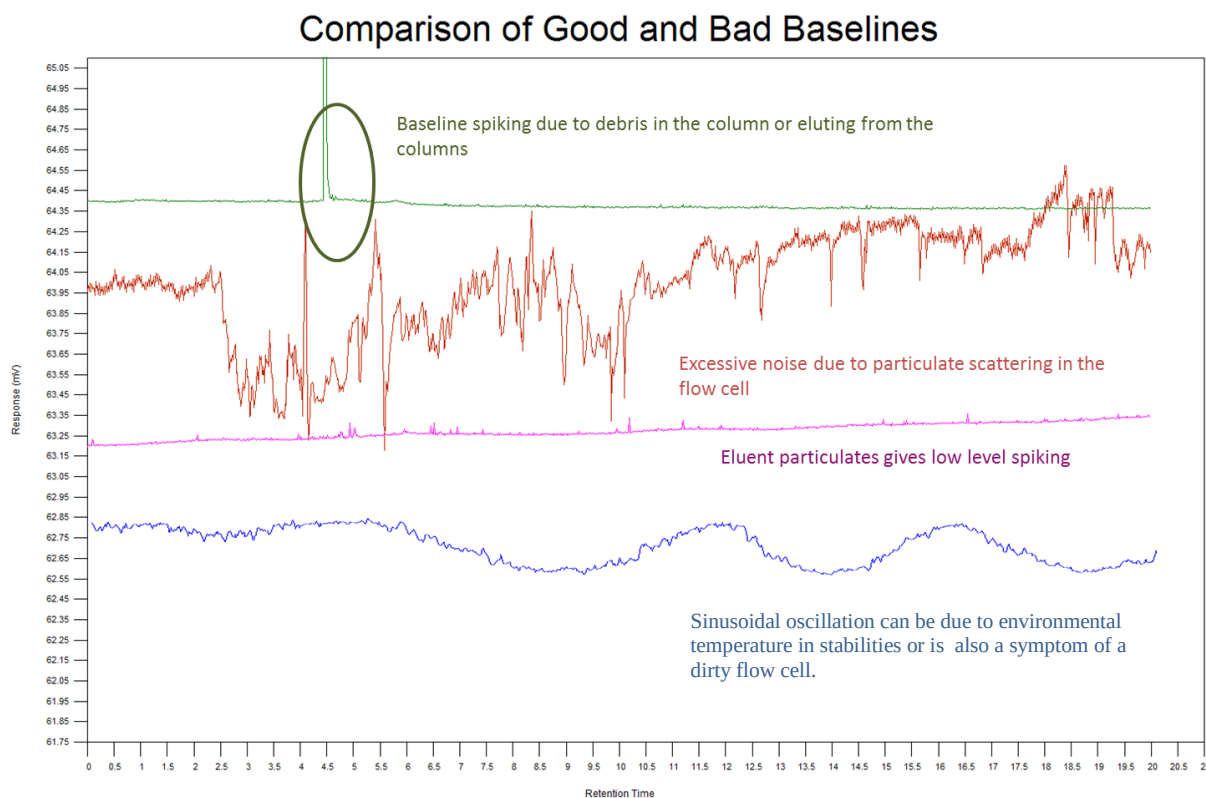
Aqueous columns – Aqueous columns contain more fines than organic columns and extra flushing is likely to be required, at least overnight.

3. **Changing Pump Flow rate** – Fines can be easily dislodged from the chromatography column when the pump flow is turned on. The faster the flow rate is set, the harder the column bed is shocked by this flow rate, the greater number of particles that are dislodged from the column. It is therefore recommended that the flow rate is gradually increased and decreased. For a majority of chromatography systems it is possible to program the pump to automatically increment the flow change, set at 0.5 ml/min/min. Gradually decreasing the flow rate is also recommended as good practice. If no ramp gradient can be fixed, then the pump should be initially set at 0.1 ml/min and the pressure on the system allowed reaching equilibrium before the flow increased to 0.2 ml/min. Adjusting at 0.1 ml/min increments only, the flow rate should be slowly increased to the desired flow rate.
4. **High baseline offset and/or spiky baseline** – This is the result of contaminating your light scattering flow cell with particulates which may be resolved by continually flushing of the system with filtered solvent and careful operation of the pump. If stringent lab conditions have not been met for a time then the flow cell may need to be dismantled and cleaned.
5. **In-line filters** – To help prevent particulates that originate from the solvent and/or the column entering the flow cell it is good practice to use an inline filter fitted directly after the column set. These inline filter assemblies (part# PL0660-0002 for organic and part# G7808-64001 for Bio-SEC applications) are fitted with 0.2 µm membranes to prevent the larger dust particles from entering the flow cell but not restricting the sample of interest. please refer to the below list of filters
0.2µ organic membrane VWR part# 514-4186 or Pall Corporation part number 6614, 100/pk.
0.2µ aqueous membrane VWR part# 514-4161 or Pall Corporation part number 60298, 100/pk.
6. **Filtering Solvents** – If using Suction Filter Device Apparatus for filtering solvents also known as “Büchner funnel” please refer to the below list of filters
Agilent part# 3150-0509 PTFE filter membranes 0.45µ, 47mm, 10/pk
Agilent part# 9301-0845 Nylon filter membranes 0.45µ, 47mm, 100/pk.
Whatman Part# 6809-5002 Anodisc membrane filters 0.02 µ, 47mm, 50/pk.
PALL part# 60310 or VWR Part# 514-4166 membrane filters 0.1 µ, 47mm, 100/pk.

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7. **Other symptoms of cell contamination** - There are several reasons why a light scattering flow cell would need cleaning and the most common symptoms are high baseline noise and/or the light scattering signals off-scale. Sinusoidal oscillation on the light scattering baselines (if not temperature related) is also a strong indication that the cell is dirty and requires cleaning.

As a visual indication of good and bad baselines see the graph below. This shows some of the symptoms that are prevalent with light scattering and the common reasons for such behavior.



The four most common cases for the cause of the poor baselines shown above are typically:-

- **Case 1** Particulate matter from columns
- **Case 2** Visible particles floating in the cell that do not exit easily after continuous pumping

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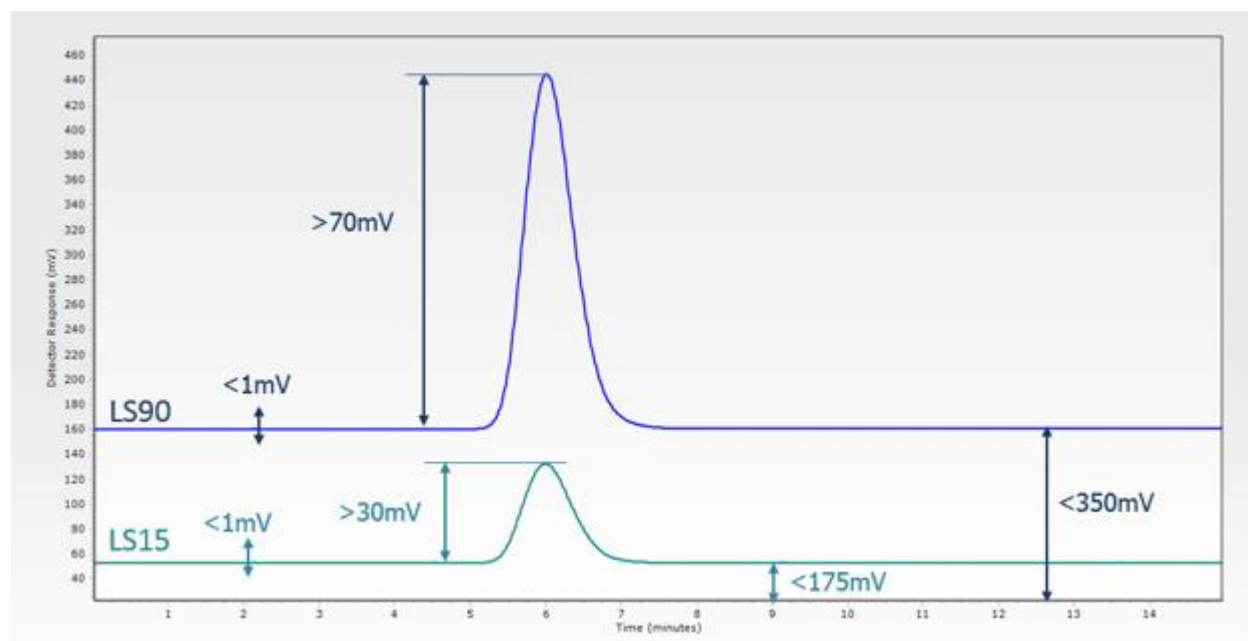
- **Case 3** Salt crystals deposited in the cell and lens walls after the introduction of an inappropriate solvent following use of a salt buffer,
- **Case 4** Dried sample and other material when the cell has been allowed to run dry and the solvent has evaporated

On most occasions, all of these cases can be resolved by continuously pumping eluent through the detector to dislodge or dissolve the offending scattering entity, but in the worst case the flow cell will have to be dismantled and cleaned.

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4.0 QC DATA

The results below show the typical QC test carried out on all the Agilent light scattering detectors with the indicated pass specifications. The test probe is a polystyrene standard $M_w \sim 260\text{Kg/mol}$, PDI 2.5 (Part#: PL2010-0000) dissolved in THF. The solution is prepared at 1mg/ml and $100\mu\text{l}$ injected onto a PLgel MIXED-C column with a 1.0ml/min flow of THF.



5.0 SOLVENTS

It should be remembered that the light scattering offset is a direct measurement of the scattering of the solvent or eluent and if the solvent is “dirty” and contains particulates then the scattering will be higher. The table below, compiled from empirical data should be used as a general guideline to the **expected offsets** for a few commonly used eluents. The best performance and the lowest solvent scattering is general obtained when the eluent has been filtered through a $0.02\mu\text{m}$ (best, or $<0.2\mu\text{m}$ is acceptable) 2-3 times.

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Table of typical solvent scattering values under conditions specified in the comment column.

Eluent	LS90 Baseline	LS15 Baseline	Comments
THF	<350mV	<175mV	1ml/min flow, (HT-LS only offset pots set to max and divide by 10 box fitted)
TCB	<600mV	<300mV	1ml/min flow, (HT-LS only offset pots set to max and divide by 10 box fitted)
Water	<50mV	<25mV	

Aqueous Eluents

Water is a particularly poor scattering media and although this has the positive affect that the baseline offset is particularly low, the negative affect is that any particle (dust) in the water scatters strongly give rise to a spikey baseline. To counteract this spikey baseline it is essential that the eluent is filtered down to 0.02µm three times, the columns thoroughly flushed to waste before being connected to the light scattering detector and potentially an inline filter fitted after the column immediately before the light scattering detector to minimize particles reaching the flow cell. The addition of buffers to the water, as long as they are fully soluble, tend to have the effect of suppressing the dust and spikiness on the baseline line but again filtration is required after the buffers have dissolved.

6.0 CELL CLEANING PROCEDURE

For users of the 1260 Infinity MDS the cell cleaning procedure is not recommended, please contact your local Agilent office. For users of the PL-GPC 220 the LS detector can be cleaned if care and consideration is taken.

1. Locate your lens cleaning kit that was supplied with your light scattering instrument.
2. Assemble the following items:-
 - a. A small glass dish, or beaker
 - b. Acetone (ultra grade, low residue)
 - c. Tweezers
 - d. Kodak lint-free lens cleaning paper

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- e. Latex gloves
 - f. Canned air (particle free)
 - g. 9/64" hex wrench
 - h. 5/16" and 1/4" open end wrench
 - i. 3/32" hex wrench
 3. Turn off the instrument and disconnect from the mains.
 4. Remove/open exterior covers as required to gain access to the optical bench.
 5. Remove the fiber optic cables from the 15, 90 and if appropriate DLS.
 6. Remove the column clips from the clamp (HT-LS only)
 7. Disconnect the plumbing from the flow cell at the unions leaving the unions attached to the flow cell.
 8. Disconnect the detector wiring from the detector boards (RT-LS units)
 9. Remove the 4 screws that secure the laser head to the optical bench and remove the laser head. (RT-LS units)
 10. On the HT-LS the laser head mounted outside the oven is connected to the flow cell via a connection tube. The tube can be decoupled from the flow cell once the baffle plate is removed that covers the laser head, the blow tube and clamp is removed and the clamps screws loosened in the oven that mount the flow cell. The optical bench can now be freely rotated and the fixing screws that attach the connection tube to the optical bench can be accessed.
 11. The flow cell can now unscrewed from the remainder of the optical bench and removed from the instrument.
 12. Using the lens removal tool included in the cleaning kit, unscrew the internal retaining nut at one end of the lens housing. Remove the spring washer. Before removing the lens retainer gently blow air around the lens to dislodge any loose debris.
 13. Very carefully remove the lens with padded tweezers. Soak the lenses in acetone (or other appropriate solvent) until all the contaminant material has been dissolved or softened so it can be wiped off easily – do not sonicate. In event that the contamination is not removed by acetone then refer to the list below for some other alternative methods and suggestions. Gently wipe both the flat end of the lens and the convex surface of the lens with a soft, lint-free lens cleaning paper.
 14. Repeat the above steps for the other lens.
 15. Carefully remove the black PTFE washers (and white PTFE washers if a HT-LS cell) from both ends of the lens housing. Avoid damage to any of the internal surfaces, particularly of the cell. They can be scratched very easily.
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16. Flush the center of the cell body with a little solvent and small soft brush.
17. Working on one side of the cell at a time started with the flanged side insert a rinsed black PTFE washer and place it into the cell (if reassembling a HT-LS a white PTFE cell going in first).
18. Ensure that no dust has been attracted to the lens – look through the curved side and clean the flat side again
19. Carefully replace the large mushroom lens the spring retainer, spring and then the retaining nut.
20. Using the tool with the cell held on the bench and tightening by just using your thumb and fore-finger. When correctly seated the inner ring of the retaining nut will be approximately flush with the lens retainer.
21. Repeat the assembly procedure on the other side and then carefully fill the cell with solvent to ensure there isn't a leak before fitting the cell back into the instrument.

For removal of salt crystals, soak in warm water – do not sonicate the lenses.

Other Solvents

- Distilled or deionized water filtered to 0.1 μm
- Isopropanol
- Methanol
- Liquid detergent
- Sterile cotton balls
- Tissue paper
- 3 plastic containers (for soaking)

Procedure:-

1. Wash the optic in detergent and water using the cotton balls
2. Immediately rinse with filtered water. Do not let the water dry before placing the lens into a bath of IPA.
3. Remove from the IPA and place in a bath of methanol
4. Remove from the methanol and using tissue soaked in methanol wipe the lens until there are no streaks.

6.0 LIGHT SCATTERING THEORY

Light Scattering refers to the process in which light from an incident polarized laser beam is scattered in all directions when it interacts with a molecule or particle. Light scattering is an

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everyday occurrence and was first described by Lord Rayleigh in the late 1800's. An example of light scattering is the scattering of sunlight by particles in the atmosphere: the sky is blue because shorter visible wavelength radiation (blue light) is scattered more strongly by the gas molecules in air than light of longer wavelengths (red light). There are two general techniques for the measurement of physical properties of polymers (synthetic polymers and natural polymers such as proteins and polysaccharides), virions, liposomes and particles.

- **Static Light Scattering** (also known as Rayleigh scattering or classical light scattering) – The intensity of the scattered light from the sample dissolved in the solvent. This difference between the two measurements can be used to determine the average molecular weight and R_g (radius of gyration). To complete the molecular weight determination, the concentration of the compound of interest is required (typically obtained from a refractive index detector or an absorbance detector).
- **Dynamic Light Scattering** (also known as Quasi-elastic scattering, Photocorrelation Spectroscopy, or Beat Spectroscopy) - The fluctuations of the intensity of the scattered light is used to determine the diffusion coefficient of the molecules as they move in solution (Brownian motion). The Stokes-Einstein equation is used to determine R_h (the hydrodynamic radius of the molecule).

Static and Dynamic measurements can be made on a sample in a cuvette or in a flowing stream such as that found in high performance liquid chromatography (HPLC), size exclusion chromatography (SEC) or gel permeation chromatography (GPC). In flowing streams, measurements are made on each elution slice using a unique light scattering design and very fast digital signal processors (which are essentially special purpose computers).

The Physical Basis of Light Scattering

Light consists of perpendicular electric and magnetic fields that oscillate in a direction that is perpendicular to the direction of propagation of the light. When light strikes a molecule, the electrons will experience a force due to the electric field and will move slightly. This movement will induce an oscillating dipole moment that will radiate light in all directions at the oscillating frequency. This radiated light is the scattered light that is detected and processed as described below.

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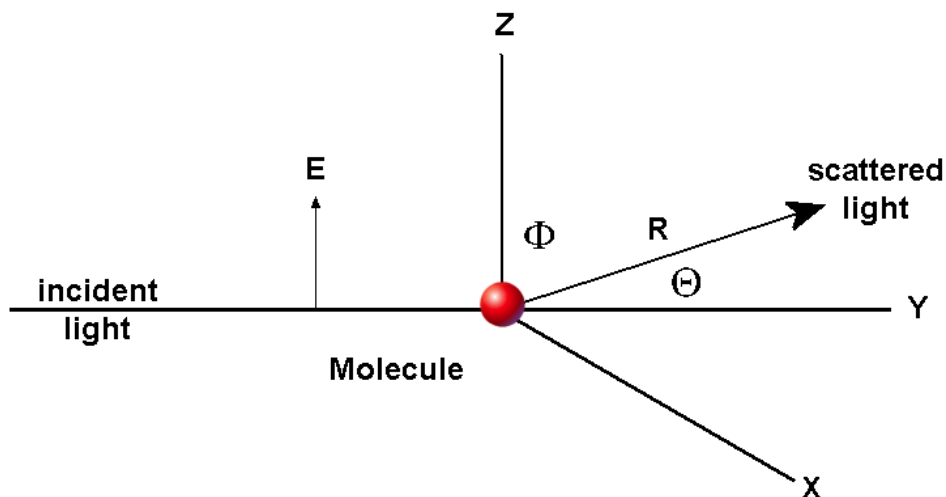


Figure 1 Light Scattered by a Molecule

Error! Reference source not found.1 describes the spatial arrangement of the incident light and scattered light in the light scattering experiment. The light is polarized in the vertical direction, thus the electric field will oscillate in the Z direction and the magnetic field will oscillate in the X direction.

A diode laser, which is a monochromatic source of light that can be focused to a very small point in the centre of the sample cell, is used in the light scattering system. Typical sources include a semiconductor laser that can provide 20 to 50mW at 655nm and another, which provides 100 to 150mW at 890nm. The scattered light is collected at a given angle and orientation (e.g. 15° or 90°) from the incident radiation, and is used to deduce the desired molecular properties.

Static Light Scattering

- Measuring the Molecular Weight

Note: Agilent, Inc. systems include polarized laser light sources, the equations presented in this chapter will be slightly different than those presented in discussions of light scattering when non-polarized sources are used.

The electric dipole moment that is induced is shown in equation 1.

$$\vec{p} = \alpha \vec{E} \quad 1$$

where: ρ is the dipole moment

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α is the polarizability

E is the electric field

The polarizability can be related to measurable parameters via equation 2.

$$A = M_w \left(\frac{dn/dc}{2 \cdot \pi \cdot N_A} \right)^2 \quad 2$$

where: M_w is the molecular weight

N_A is Avogadro's number (6.02×10^{23} molecules per mole)

dn/dc is the change in the index of refraction as a function of the change in concentration.

It is considered to be a constant for any specified solvent-solute pair under constant operating conditions.

The oscillating dipole will radiate light in all directions at the oscillating frequency. This is the origin of scattered light. If a single molecule has dimensions that are small with respect to the wavelength of the incident light, the intensity of the light can be defined by equation 3.

$$I_s = \frac{[4 \cdot \pi^2 \cdot M_w \cdot \sin^2 \Phi \cdot (dn/dc)^2 \cdot I_0]}{N_A^2 \cdot \lambda_0^4 \cdot R^2} \quad 3$$

where: I_s is the intensity of the radiated light I_s

I_0 is the intensity of the incident light

λ_0 is the wavelength of light in a vacuum

ϕ and R are as defined in **Error! Reference source not found.**

If we collect light from a volume V of a solution with a concentration c (g/ml), the intensity of scattered light can be found by multiplying equation 3 by the number of molecules in the volume V .

The number of molecules can be expressed by $N_A \cdot c \cdot \frac{V}{M_w}$

Now if we solve for M_w , we obtain equation 4

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$$M_w = \frac{[N_A^2 \cdot \lambda_0^4 \cdot R^2 \cdot I_s]}{4 \cdot \pi^2 \cdot M_w \cdot \sin^2 \Phi \cdot c \cdot (dn/dc)^2 \cdot I_0 \cdot V} \quad \text{equation 4}$$

Collecting all the constants and instrumental parameters into an overall instrumental constant, A, we obtain equation 5.

$$M_w = \frac{[I_s]}{A \cdot c \cdot (dn/dc)^2 \cdot I_0} \quad \text{equation 5}$$

Equation 5 can be used to measure the molecular weight M_w of small molecules at any scattering angle Θ . It should be noted, however, that larger molecules scatter less light at high values of Θ than at low angles because of interference effects caused by the fact that light scattered from one part of the molecule travels a different distance from another part of the molecule, and is not in phase with light scattered. This phenomenon can be quantified by defining the light scattering form factor (equation 6). A more detailed discussion of the form factor is presented in The Form Factor sub section.

$$P(\Theta) = \frac{\text{scattering intensity at angle } \Theta}{\text{scattering intensity at angle } \Phi} \quad \text{equation 6}$$

It should be noted that $P(\Theta)$ can be written as a series as shown in equation 7.

$$P(\Theta) = 1 - \frac{1}{3} (q^2 \cdot R_g^2) + \quad \text{equation 7}$$

where: $q = 4 \pi \cdot n \cdot \sin(\Theta/2) / \lambda_0$

R_g is the radius of gyration of the molecule

n is the index of refraction of the liquid

λ_0 is the wavelength of light in a vacuum

For scattering at 15° and 90° , equation 7 can be expressed as equations 8 and 9, respectively.

$$P(\Theta) = 1 - 26.3 \left(\frac{R_g \cdot n}{\lambda_0} \right)^2 \quad \text{equation 8}$$

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$$P(\Theta) = 1 - 0.0897 \left(\frac{R_g \cdot n}{\lambda_0} \right)^2 \quad \text{equation 9}$$

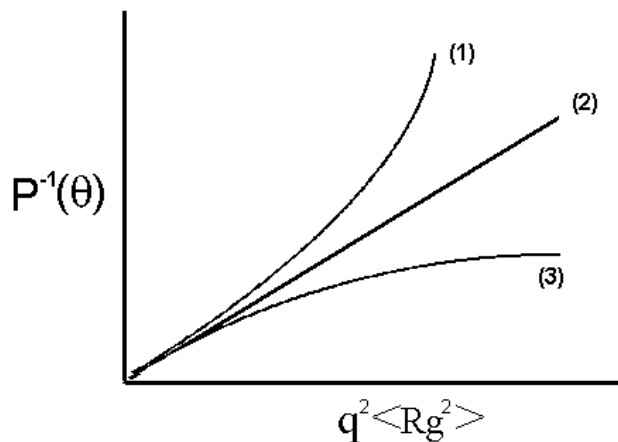
We present a table of P values for 15° and 90° degrees as a function of molecular weight (for molecules with molecular weight from 10⁴ to 10⁸ Daltons) in Table 8-1. These values assume that the molecules are random coils.

Mw	Rg (Approx) nm	P(90°)	P(15°)
5 x 10 ³	2.3	0.9993	1.0000
5 x 10 ⁴	7	0.9993	0.9998
5 x 10 ⁵	23	0.9993	0.9976
5 x 10 ⁶	70	*1	0.9780
5 x 10 ⁷	230	*1	0.7622

*1 values depend on shape

Table 1. Value of P(90°) and P(15°) for $\lambda_0 = 655\text{nm}$, $n = 1.5$

If the value of $P(\Theta)$ found in equation 7 is below 0.7, higher order components become important. In this case, $P(\Theta)$ depends on R_g and also on the shape of the molecule as shown in figure 5.2.



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**Figure 2. Values of $P^{-1}(\Theta)$ as a function of $q^2\langle R_g^2 \rangle$ for various particle shapes:
1 = Sphere, 2 = Gaussian Coils, 3 = Rods**

It is clear that all three molecular shapes yield the same value of $P^{-1}(\Theta)$ when $q^2\langle R_g^2 \rangle$ is less than approximately 1. As the value of $P^{-1}(\Theta)$ increases, the shape of the molecule clearly influences the light scattering intensity.