# HPLC Detection
## Newer Methods

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# Preface

High-performance liquid chromatography (HPLC) has been one of the most important analytical methods used both in industry and academe. A large number of detection methods are available for detecting the separated solutes, and several of these methods have been used for a long time. Hence, the theory and application of these conventional HPLC detection methods have been discussed in detail in a large number of excellent monographs, review articles, and reports. Some of the more conventional detection methods, such as UV/vis absorption, fluorescence, and conductivity detection, are the detection methods of choice for most analyses. However, with the advent of several new developments in analytical chemistry, new detection methods have become available as HPLC detectors. For example, long-lived luminescence under a variety of experimental conditions may be advantageous as an HPLC detection mechanism that significantly lowers background interference. Also, Fourier transform infrared- (FTIR) and mass-spectrometric techniques have been employed to detect HPLC eluents. From these and other advances in analytical chemistry, a number of novel HPLC detection methods have been developed. In the area of bioanalytical chemistry, several reports have appeared in which long-wavelength fluorescence using the extremely cost-effective semiconductor laser excited fluorescence has been employed to detect biologically important molecules in the HPLC eluent.

The primary purpose in writing this book was to give a rather detailed survey of these new, less conventional detection methods. It is not the goal of this book to provide comprehensive coverage of the detection methods used in HPLC separations; several excellent monographs have already accomplished this. For the sake of brevity, several of the most widely used methods and detectors have been inten-

tionally omitted. Accordingly, the reader will not find detailed discussions about conventional UV HPLC detection or regular fluorescence. However, emphasis has been placed on the more modern or just developing detection methods and their use in HPLC separations. This book is intended to serve the needs of those chromatographers whose work is restricted by the limits of conventional detection methods. It will be of interest to those working in HPLC separations and those who have a more specialized interest in HPLC detection. The book should also find use as a reference text or supplemental material for courses involved with separation analysis techniques.

In Chapter 1 measurement concepts are presented for detection in micro-HPLC separations using lasers. In Chapter 2, the advantages of using long-lived luminescence detection methods are discussed to illustrate its applications with trace concentrations. The utility of chemiluminescence in HPLC detection is presented in Chapter 3. Chapter 4 discusses near-infrared semiconductor laser fluorescence, one of the latest emerging detection methods for ultratrace concentrations. The somewhat more conventional electrochemical detection method is discussed in Chapter 5, however, with a special emphasis on less conventional applications.

Chapter 6 serves as an introduction to the second part of the book, which discusses powerful photothermal detection methods. This detection method has significant utility at extremely low concentrations. The last three chapters focus on detection methods that are supplying a wealth of information about the analyte molecule in the detector. These methods may supply enough information for complete identification of the solute, hence improving the utility of HPLC. Chapter 7 discusses HPLC detection using FTIR spectroscopy. Chapter 8 gives a detailed summary of one of the most powerful detection methods, HPLC mass spectrometry. Finally, Chapter 9 is a peek into the future, indicating how the ultimate power of NMR may be combined with HPLC.

I would like to convey my most sincere appreciation to the authors of the chapters of this book. My appreciation is also extended to Dan Adams for his expert assistance on style and readability, and Dianne Becht for help in the final formatting of the book.

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# Contents

CHAPTER

# 1

# Measurement Concepts and Laser-Based Detection in High-Performance Micro Separations

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## 1.1. Purpose

High-performance micro separations combine with laser-based detection for maximum analytical performance, which is demanded in such areas as pharmaceuticals, biomedicine, and environmental science. The unique combination of laser radiation and micro separation techniques has as its basis the so-called analytical figures of merit. We devote this chapter to the measurement fundamentals of this group of microanalytical detection schemes. We shall also attempt to outline the current state of commercial development of these techniques.

## 1.2. Introduction

### 1.2.1. The Instrument Response Function and Figures of Merit

Probably the most important aspect of laser detection for micro separations is the instrument response function, as expressed by the degree of instrument (detector) response per analyte concentration or mass. Figure 1.1 shows a representative response function. Note that the axes extend over several orders of magnitude and

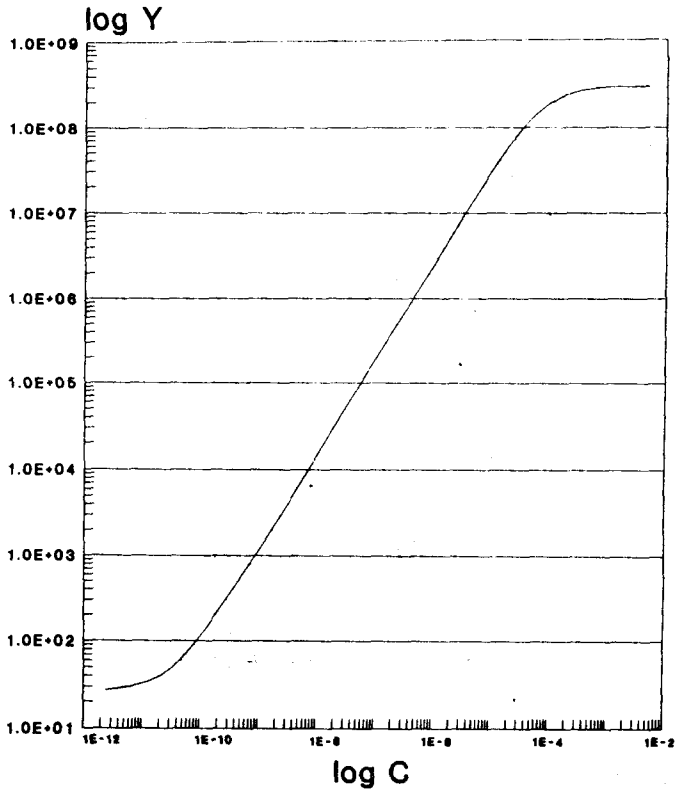


**Figure 1.1.** Instrument response function as log $Y$ versus log $C$, where $Y$ is the instrument response and $C$ the concentration of analyte. Analyte mass, $M$, may be substituted for $C$. The dotted line indicates a "rollover" effect.

that there are nonlinear portions at both low and high analyte concentration (or mass). The overall instrument response function, which includes these nonlinear regions, is obtained through curve-fitting techniques. The linear portion of the response function[1] is given by

$$Y = AC^r, \tag{1.1}$$

where $Y$ is the instrument response (volts, microamps, etc.), $A$ is a constant that is characteristic of the particular measurement, $C$ is the concentration (or mass), and $r$ is the response index. Taking the logarithm of both sides and simplifying, we have

$$\log Y = \log A + r \log C, \tag{1.2}$$

where the ordinate intercept is log $A$ and the slope is $r$. Deviation of $r$ from unity implies that the instrument response–concentration (or mass) relationship is skewed

positively ($r > 1$) or negatively ($r < 1$). What is ideal about the condition $r = 1$? Simply, that we would expect a 10- or 100-fold increase in response for a 10- or 100-fold increase in analyte concentration (or mass). This direct proportionality implies perfect correlation between input and output (i.e., chemical information and analytical signal.).[2] Overall, the instrument response function is sigmoidal, with a positive deviation from linearity at low concentration (mass) and negative deviation at high concentration (mass). These deviations are due to both measurement and instrument characteristics, such as analyte photophysics and the arrangement of electronic components within the instrument. These aspects will be discussed in the following. While the entire measurable range would include these deviations, the analytically useful portion is restricted to the linear region.

The range in concentration (or mass) over which this response function is linear is known as the *linear dynamic range*. It is properly defined as the range in analyte concentration (or mass) for which the response index is between 0.98 and 1.02. In other words, we restrict the direct proportionality between analyte concentration (or mass) and instrument response to ±2% deviation from 1 for an "operational" convenience. The linear dynamic range is properly determined using statistical routines whereby limits can be placed on the slope ($r$) and the largest abscissa range that satisfies this criterion is found.

The instrument response time is another important feature of laser-based detection. The time required for the output signal to increase or decrease to $1/e$ of the maximum response value is the response time. With no input signal the output will lag slightly behind an input signal pulse ("on"), as shown in Figure 1.2. Similarly, the output will lag temporally behind an "off" transition at the input. While this is classically attributed only to transducers, this response characteristic should be ascribed to transducers plus their associated electronics. The nature of this "signal processing" by detectors and their electronics can be linear or exponential with respect to time. Typically, analog electronics display exponential signal–time relationships, while digital electronics show a linear dependency.[3]

The slope of the calibration curve, a selected, small portion of the response function, is defined as the *calibration sensitivity*. If the sensitivity is compensated by the measurement precision, then it is referred to as the *analytical sensitivity*.[4] Presumably, if one had constructed a response–concentration calibration, then the slope of this plot would be called the *concentration calibration sensitivity;* likewise, for response–mass calibration, the slope would be the mass calibration sensitivity. The important concept here is the degree of proportionality between ordinate and abscissa for both these plots. Simply, the degree of change in these quantities is the sensitivity. Obviously, the greater the slope, the greater will be the change in ordinate value per abscissa increment. The utility of calibration and analytical sensitivities in analytical separations stems from the fact that no two analytes will have identical sensitivities, owing to differences in detector response and separation efficiency and resolution. The bottom line is that each analyte should be separately calibrated.

As one approaches the lowest response levels of the instrument, one encounters a nonlinear portion, which determines the limit of detection. The limit of detection is

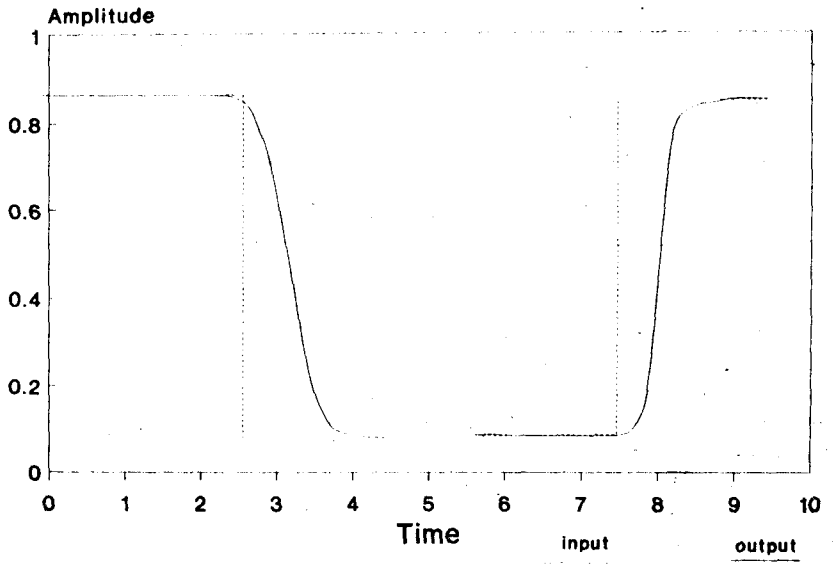


**Figure 1.2.** Temporal response of a detection system to negative and positive transients. ··· input signal; ——, output signal.

a parameter that is defined as the minimum distinguishable signal that is two or three times higher than the standard deviation of the baseline noise.[4,5] There is some disagreement[5] as to whether there must be linearity as one approaches the detection limit; hence, the detection limit might be roughly equal to the minimum concentration (or mass) in the linear dynamic range computation. For safety's sake, one would choose three standard deviations as the limit of detection, and this roughly corresponds to this point in the linear dynamic range. In any case, it is generally agreed that detector or baseline noise determines the limit of detection. Detector noise is largely independent of the type of source used. Laser-based detectors often display higher signal-to-noise levels, thus enabling lower detection limits to be realized, mostly because of the greater signal that is generated rather than by any reduction in noise.

These parameters—instrument response function, linear dynamic range, response time, calibration and analytical sensitivity, and limit of detection—are commonly referred to as *analytical figures of merit*. It is well known that lasers are ideal spectral sources for detectors and that micro separations are ideal analytical separation techniques, each in terms of its performance criteria. Yeung has eloquently and completely presented this case.[6,7] Logically, there should be unique aspects to the analytical figures of merit for laser-based micro separation techniques. Figure 1.3 shows the attributes of both the source (laser) and separation technique that influence the analytical figures of merit. More important, however, Figure 1.3 shows the

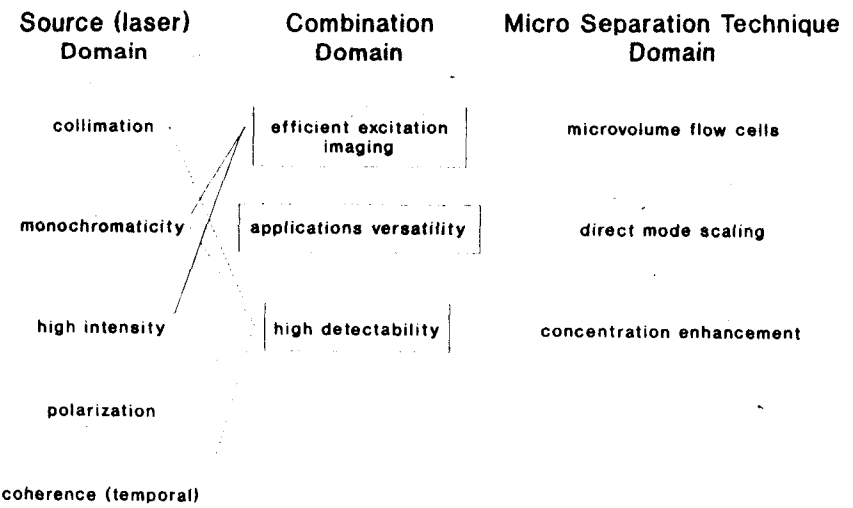


**Figure 1.3.** Comparison of attributes for the laser source, micro separation technique, and the combination.

combinations of attributes that influence detection performance with laser-based micro separation detectors. Especially unique to micro separations is the need for microvolume flow cells, an attribute that combines very well with the collimation, monochromaticity, and high intensity of the laser to provide for very efficient excitation imaging. Finally, applications versatility results when all the laser source attributes are combined with the fact that micro separations are a miniaturized version of the conventional (larger) equivalent. Indeed, one simply has to use the same sorbent and mobile phase in the miniaturized separation as was required in the large-scale version.[8] Selectivity and capacity ratios are mostly equivalent, allowing the same type of separation to be accomplished but on a much smaller (micro-analytical) scale. High detectability is an attribute of the combination that is due to the monochromaticity, collimation, high intensity, polarization, and coherence of the laser source, combined with the concentration enhancement achievable with microscale analytical separations. Specific examples of these attributes and their impact on the analytical figures of merit will be discussed in the following.

### 1.2.2. Concentration and Mass Sensitivity

There are other analytical figures of merit related to sensitivity that are intended to quantify the mass and concentration response of detectors. Scott presents the terms *detector sensitivity* and *mass sensitivity* of chromatographic detectors, referring not to the slopes of the appropriate calibration plots but to the limits of detection to which the instrument responds to concentration and mass.[9] This argument is based

on the fact that the signal-to-noise ratio is highest at the apex of a chromatographic peak, where presumably the concentration or mass flux is also highest. Because of the very small elution volumes, connecting tubing and flow cells of micro separation techniques, there is "increased mass sensitivity of concentration-sensitive detectors" in these techniques.[10] There need not be confusion, when one realizes that some detectors respond more to number density of analyte and others to number or mass flux of analyte. Since concentration and mass are interconvertible through the volume, mass sensitivity depends on concentration sensitivity to a large degree. With micro separations one would like to preserve, as much as possible, the concentration response of detection because solute mass becomes vanishingly small approaching the detection limit.

A fundamental constraint to all concentration-sensitive detection strategies is the path length, as determined by Beer's law. The illuminated volume (as determined by

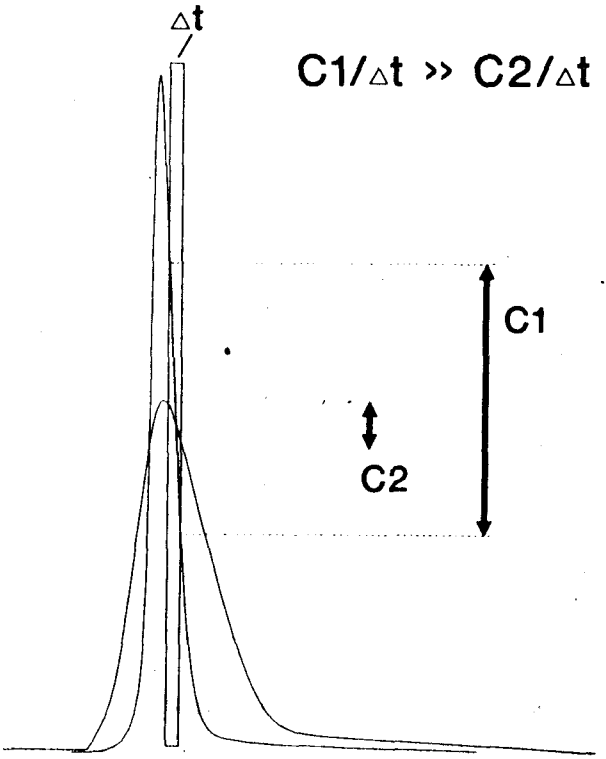


**Figure 1.4.** Illustration of enhanced mass sensitivity in high-efficiency separations using concentration-sensitive detection. The more efficient chromatogram shows a larger concentration difference, $C_1$, hence a larger mass flux of analyte during the sampling interval, $\Delta t$.