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# Uncertainty in modern spectrophotometers

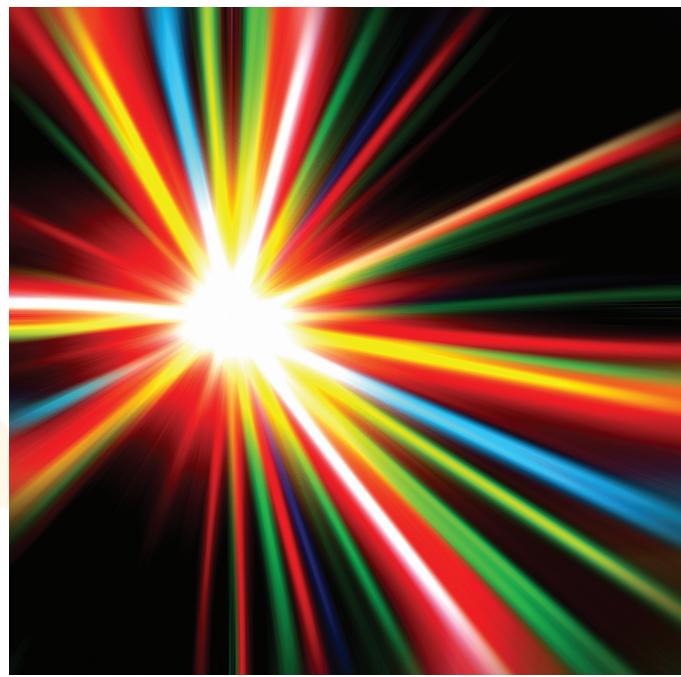
An up-to-date view of UV-vis molecular absorption instruments and measurements.

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The most authoritative studies on spectrophotometric uncertainty were done ~30 years ago by Rothman, Crouch, and Ingle (1). They dealt with the different ways in which spectrophotometric noise (the old term for uncertainty) originated and gave equations that could help buyers and users of spectrophotometers to get the lowest possible uncertainty values.

Oddly enough, the researchers found that for low absorbance values the main cause of instrumental photometric uncertainty (when standards and samples were measured) was the cell positioning. The authors recommended that "some attention should be directed toward improving cell-positioning precision in spectrophotometric measurements." When care is taken by the operator, cell-positioning uncertainty depends primarily on the cell-holder design. Thus, the authors noted that "continuous improvement in sample cell-positioning uncertainty is occurring in commercial spectrophotometers, and such improvements should eventually lead to realization of the maximum precision inherent in the commercial instruments."

That paper is still considered the most authoritative study on the subject (2–4), and no new relevant studies seem to have been undertaken, even though the instruments have evolved. In fact, we have been unable to find any study that includes uncertainty data for absorbance values <0.1. The same errors, with the same relative significance, are assumed. Thus, some questions naturally arise when buying a modern UV-vis spectrophotometer or when checking its performance. Is this degree of uncertainty commonplace in present-day laboratories? Have the new spectrophotometers introduced significant improvements in cell-holder design? Apart from classical detectors (thermal, phototubes, photomultiplier-type, etc.), different detection systems have been developed, such as diode arrays and CCDs. And new ways of measuring have appeared, such as flow and sequential injection (SI) and LC- and CE-diode-array detectors. Do the same causes of error apply to these instruments and systems, and in the same manner? Which components of spectrophotometers



are expected to generate significant uncertainty?

This product review discusses the relevance of different kinds of spectrophotometric uncertainty, with special emphasis on the low-absorption region. Close attention is paid to cell positioning, which is supposed to be the most important source of uncertainty at low absorbances. This article covers some modern photomultiplier-type spectrophotometers and diode-array instruments, and mathematical equations that explain the experimental behavior are given. Numerical values for the different uncertainty constants are also given. An automated way of taking samples to the detector, the SI system, in which the cell is not moved between measurements, is also considered.

The results show that uncertainty has appreciably diminished during the past 30 years, probably because of improvements in the instruments themselves and in cell-holder designs. For the diode-array detector, shot-noise constants could be as much as 1 order of magnitude below the literature value of  $3 \times 10^{-3}$  that is still accepted. Under customary working conditions, diode-array detectors exhibited the lowest uncertainty levels. If the original cell-holder conditions are maintained, the cell-positioning effect can be practically irrelevant in the combined experimental uncertainty. In flow systems, special care should be paid to the injection step, because it can easily become the dominant contributor to the uncertainty.

**Table 1. Sources of uncertainty in photometric measurements.**

Source of photometric error	Expression	Relative uncertainty in absorbance	Equation
General expression of photometric error	$\frac{s_C}{C} = \frac{s_{\text{Abs}}}{\text{Abs}} = \frac{0.434}{T \log T} s_T$	$\frac{s_{\text{Abs}}}{\text{Abs}} = \frac{0.434}{\text{Abs}} \frac{s_T}{10^{\text{Abs}}}$	1
Thermal detectors	$s_T = k_1 \sqrt{1 + 10^{-2\text{Abs}}}^a$	$\frac{s_{\text{Abs}}}{\text{Abs}} = \frac{0.434}{\text{Abs}} k_1 \sqrt{1 + 10^{2\text{Abs}}}^b$	2
Photon detectors	$s_T = k_2 \sqrt{10^{-\text{Abs}} + 10^{-2\text{Abs}}}$	$\frac{s_{\text{Abs}}}{\text{Abs}} = \frac{0.434}{\text{Abs}} k_2 \sqrt{1 + 10^{\text{Abs}}}$	3
Cell positioning and source fluctuations	$s_T = k_3 10^{-\text{Abs}}$	$\frac{s_{\text{Abs}}}{\text{Abs}} = \frac{0.434}{\text{Abs}} k_3$	4
Combined photometric error		$\frac{s_{\text{Abs}}}{\text{Abs}} = \sqrt{\sum_i \left( \frac{s_{\text{Abs}}}{\text{Abs}} \right)_i^2}$	5

<sup>a</sup>Sometimes the proposed expression is  $s_T = k_1$ . The two expressions become similar for high absorbance values.

<sup>b</sup>When  $s_T = k_1$ , Equation 2 becomes:  $\frac{s_{\text{Abs}}}{\text{Abs}} = \frac{0.434}{\text{Abs}} k_1 10^{\text{Abs}}$

## Uncertainty and its sources

Experimental measurements are subject to uncertainty from different sources, such as the operator, the instrument, and ambient conditions. The combined uncertainty will be a consequence of all of them and will be included in the experimental signal. Some kinds of uncertainty increase as the experimental signal does, whereas others do not. This is probably the reason that S/N is frequently used to express uncertainties, in which the noise (N) includes every kind of uncertainty. One of the most important performance specifications of these instruments is the inherent level of uncertainty, because the precision of measurements will depend upon it.

The sources of uncertainty in UV-vis spectrophotometers have been very well known for many years. All of them obey Equation 1 (Table 1). Depending on the uncertainty source, Equations 2–4 may also be applicable (Table 1; 1–5). The uncertainty generated in the detector depends on the absorbance, but the uncertainties generated by the flickering of the light source or by imprecise cell positioning do not. Obviously, a lower absorbance leads to greater uncertainty in the combined precision. The uncertainty in the photometric measurements dictates the lowest significant value of absorbance that can be measured and, consequently, influences the limit of detection and all the figures of merit (6). Considering that the additive form of uncertainty is the variance, the combined uncertainty can be obtained through Equation 5 (Table 1). As a consequence of Equation 5, only the major factors in it (the sources that are numerically greater) will be significant—this is called the squares effect.

Different types of uncertainty apply to the various instruments. Thermal detectors can be characterized by Equation 2,

whereas uncertainty in photon detectors obeys Equation 3. Equation 4 will have to be considered when cell positioning and source fluctuations come into play. The value of constants  $k_1$ ,  $k_2$ , and  $k_3$  (Table 1) can be used as an indication of the expected precision of an instrument. These values can be obtained by taking consecutive measurements (say, >20) of a suitable absorbent solution, at one or several wavelengths. The cell may be kept in the cell holder ( $k_1$  and  $k_2$ ) or removed and put back between measurements ( $k_3$ ). In this last case, Equation 4 is included in the combined effect, and the value of  $k_3$  can be obtained whenever the value of  $k_1$  or  $k_2$  is known in advance.

**Table 2. Experimental values for the constants that rule the spectrophotometric uncertainty.<sup>1</sup>**

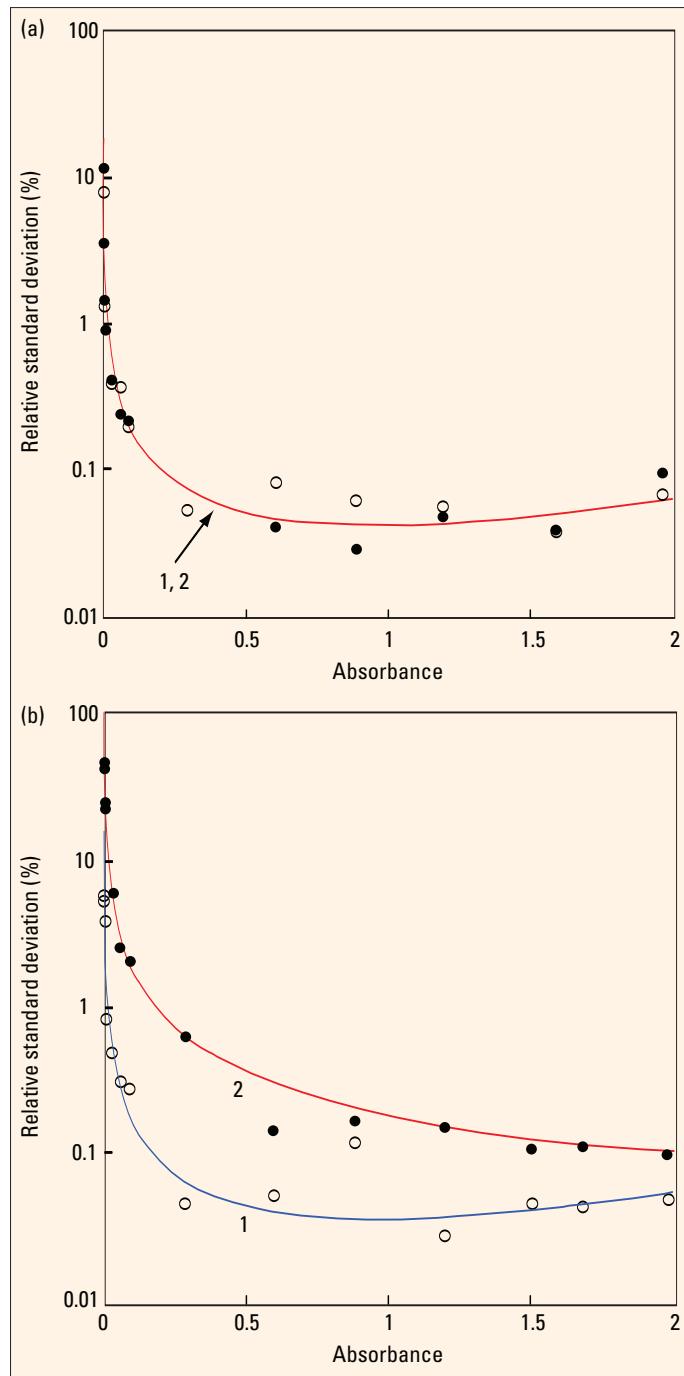
Type of detector	Absorbent	$k_2 (\times 10^4)$	$k_3 (\times 10^4)$
Diode array (HP 8452A)	Dichromate	3.0 (0.2)	0.2 (5)
	Ferroin	2.3 (0.1)	2.0 (0.6)
	Dichromate <sup>2</sup>	2.6 (0.2)	42 (1)
Photomultiplier 1 (PerkinElmer Lambda 5)	Dichromate	7.6 (0.3)	12.4 (0.2)
	Ferroin	4.5 (0.2)	27 (1)
Photomultiplier 2 (PerkinElmer Lambda EZ201)	Dichromate	7.2 (0.1)	4.5 (0.7)
	Ferroin	7.7 (0.4)	Negligible
Diode array (SI) <sup>3,4</sup>	Ferroin	2.3	

<sup>1</sup> Constant values were obtained by nonlinear regression analysis; the standard deviation is given in parentheses. The absorbance range is 0–2.0; all spectrophotometers conformed to specifications.

<sup>2</sup> Old cell holder used.

<sup>3</sup> SI system consists of an autoburette with Hamilton syringes and an eight-port selection-valve module.

<sup>4</sup>  $k_1 = 2.4 (0.1)$ .



**FIGURE 1.** Diode-array spectrophotometer performance.

Data for (a) new cell holder and (b) 10-year-old cell holder. Open circles indicate data when the cell is not moved between measurements; filled circles indicate data when the cell is moved between measurements. Line 1, simulated behavior of shot noise; line 2, simulation of the combined effect of shot noise and cell positioning.

### Diode-array spectrophotometers

For these experiments, we chose dichromate solutions of different concentrations (in  $5 \times 10^{-3}$  M sulfuric acid) as the absorbent ( $\lambda = 350$  nm). Figure 1a shows a plot of the relative uncertainty versus absorbance and a nonlinear regression anal-

ysis. When the cell is not moved between measurements (open circles), the behavior of uncertainty can be explained by Equation 3 (shot noise);  $k_2$  was estimated to be  $3.0 \times 10^{-4}$  (Table 2). This was the general way in which the value of uncertainty was determined as the work proceeded.

When a dichromate solution with a concentration as low as  $4.7 \times 10^{-7}$  M was used at different wavelengths between 275 and 525 nm (different absorptivities),  $k_2$  randomly ranged between 2 and  $3 \times 10^{-4}$ . These results agree with the value given in Table 2. In this case, however,  $k_2$  will not be very robust, although it can be used to check other measurements.

If the cell is taken from its holder before each measurement, the results in Figure 1a (filled circles) are found. These results should fit Equation 5 to yield

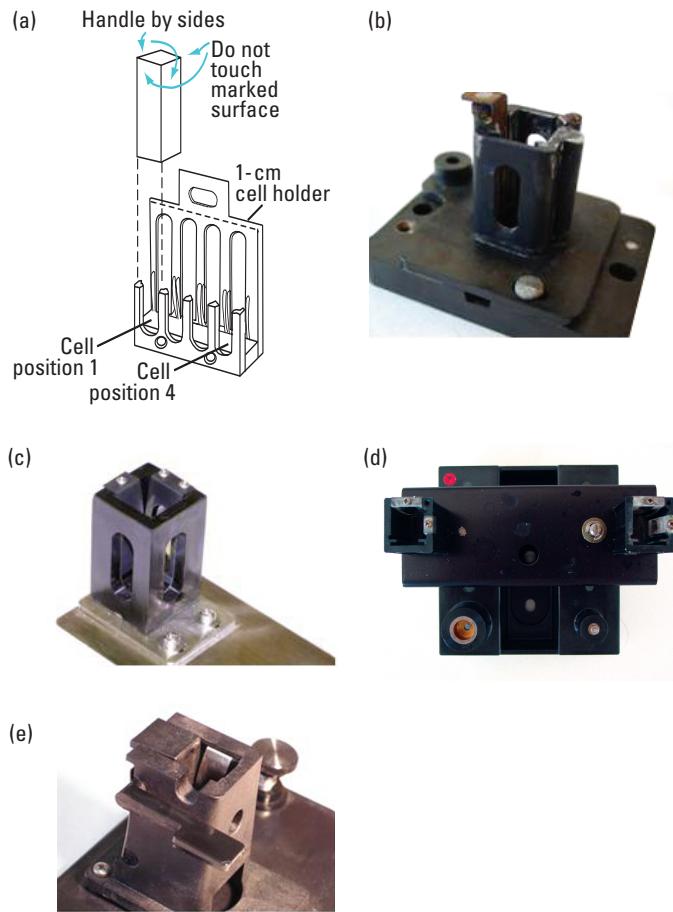
$$\left( \frac{s_{\text{Abs}}}{\text{Abs}} \right)_{\text{combined}}^2 = \left( \frac{s_{\text{Abs}}}{\text{Abs}} \right)_{\text{shot noise}}^2 + \left( \frac{s_{\text{Abs}}}{\text{Abs}} \right)_{\text{cell positioning}}^2 \quad (6)$$

The  $k_2$  previously obtained can be used to estimate  $k_3$  from Equation 6; in this case,  $k_3 = 0.2 \times 10^{-4}$ .

The mathematical form of Equations 3 and 4 and the effect of the squares in Equation 6 make the significance of Equation 4 on the combined effect practically negligible. The exception is when  $k_3 \gg k_2$ ; this is the only case in which the term that corresponds to cell positioning will be the limiting expression of uncertainty. The low value of  $k_3$  (<10% of  $k_2$  in this case) means that the effect of cell positioning on the combined effect is negligible. That is the reason the fit is nearly the same (Figure 1a, curves 1 and 2) regardless of whether the cell is moved. It can be concluded that, at least for the cases considered, shot noise is the limiting form of uncertainty and the cell-positioning effect is negligible, even for low absorbances. This is contrary to what has been assumed historically. Moreover, typical values given for  $k_2$  in the literature ( $\sim 0.003$ ; 3) are  $\sim 1$  order of magnitude higher than those found in this work (Table 2) for a diode-array spectrophotometer.

Table 2 also gives the results when an absorbent in the visible region, such as ferroin (water solution,  $\lambda_{\text{max}} = 514$  nm), was used. In this case,  $k_2$  ( $2.3 \times 10^{-4}$ ) and  $k_3$  ( $2.0 \times 10^{-4}$ ) were similar, but even so, the effect of cell positioning has very little practical relevance.

The results completely changed when a 10-year-old (but still in use) cell holder was substituted. Figure 1b shows that shot noise (open circles) is similar to that previously found, but the combined uncertainty (filled circles) is much higher for the old cell holder. The differences are due to the strong effect of cell positioning in the old cell holder. The  $k_3$  value ( $4.2 \times 10^{-3}$ ) is  $\sim 2$  orders of magnitude higher than the one found for the new cell holder (Table 2); however, it is still lower than the typical values given in the literature for this effect (0.013 for quality spectrophotometers; 1, 3). The reason is that cell-holder design has evolved during the past 30 years. Instrument vendors have improved both the fitting of the cell in the cell holder and the attachment of the cell holder in the framework. In any case, the cell-holder position should be as reproducible as possible.



**FIGURE 2.** Cell-holder designs.

(a) McPherson EU-701A, (b) PerkinElmer Lambda 5, (c) Shimadzu UV-260, (d) PerkinElmer Lambda EZ201, and (e) Hewlett Packard HP 8452A. [(a) reprinted with permission from GCA McPherson.]

Originally, cells were fit tight to the cell holder through some kind of iron straps. For example, the spectrophotometer used in Ref. 1 (EU-701 from GCA McPherson Corp.) had a four-position cell holder furnished with thin iron straps, which acted as a spring, and had a Plexiglas base to hold the cell in position (Figure 2a). Over time, the strap design became wider, and the cell holder of the PerkinElmer Lambda 5 furnished one wider iron strap at a 45° angle (Figure 2b). Afterward, two metal bands were used to get a more reproducible cell position; these systems were very popular for years (Figures 2c and 2d, which correspond to the Shimadzu UV-260 and PerkinElmer Lambda EZ201, respectively).

Today, it seems that more-efficient systems are used, such as the one in Figure 2e that includes both a metal band and a lever (Hewlett Packard HP 8452A), although some simple systems are also used. On the other hand, softer materials (plastic) allow a tighter and more reproducible fitting and less strain. Special care should be taken with these materials because small spills and splashes of liquid, especially from acidic solutions, easily deteriorate them and degrade the fitting of the cell.

### Photomultiplier spectrophotometers

The method discussed earlier was used to determine  $k_2$  and  $k_3$  for two photomultiplier spectrophotometers (Table 2). The Lambda 5 was an old, high-quality, and much-used spectrophotometer; the Lambda EZ201 was a new, simple, and not-much-used instrument. Both of them showed a similar shot-noise level, and the values found for  $k_2$  ( $4\text{--}8 \times 10^{-4}$ ) were 2–3-fold higher than those for the diode-array spectrophotometer but still much lower than those given in the literature ( $k_2 = 0.003$ ; 3).

These differences are probably due to several improvements in instruments that affect both low and high absorbances. Improvements in electronics and signal treatment have allowed more-stable radiation sources, which have reduced the instrumental noise mainly at low absorbance values. In addition, noise related to the amplification and integration steps (carried out currently with digital instead of analog filters) has also reduced the overall noise for high intensity values.

The evolution of instrumentation for UV-vis spectrophotometry through 1985 was reviewed in detail by Altemose et al. (7, 8). The generalization of the double-beam spectrophotometer, the incorporation of two lamps (deuterium and tungsten), the appearance of silicon chip detectors, and the introduction of microprocessors for spectrophotometer control and data treatment (averaging, smoothing, spectra derivation, etc.) were highlighted. Since then, most of the improvements deal with light emission stabilization, the emergence of CCD and photodiode array detectors, elimination of the mobile components, and amplification-noise reduction (9, 10), in such a way that the limit of detection for absorbance measurements made with current research-grade commercial spectrophotometers is  $5\text{--}6 \times 10^{-5}$  absorbance units (11).

Cell positioning also provided significant results in these spectrophotometers. For the Lambda 5,  $k_3$  was high enough to make cell positioning the limiting part of the uncertainty whenever the absorbance was low enough (~1.5 for dichromate and ~0.25 for ferroin). Nevertheless, for the Lambda EZ201,  $k_3$  was low enough to render cell positioning negligible. As a whole, the most important difference between the instruments (as far as uncertainty is concerned) was not the inherent quality but the condition of the cell holder.

### SI systems

Figure 3 plots the standard deviation versus absorbance when an SI system is used (filled circles). The way in which standard deviation increases for high absorbances points to an effect independent of the strength of the signal arriving at the detector (footnotes in Table 1). The detector was the same diode-array spectrophotometer used earlier (Figure 1), and ferroin was used as the absorbent. An independent study of the detector shot noise in batch samples is also presented in Figure 3 (open circles). Differences between the two sets of data should be attributed to the SI system (excluding the detector); the injection step probably dominates, because no cell-positioning effect occurs. From Equation 5, the following expression should be obeyed

$$\left(\frac{s_{\text{Abs}}}{\text{Abs}}\right)^2_{\text{combined}} = \left(\frac{s_{\text{SI}}}{\text{Abs}}\right)^2_{\text{injection, etc.}} + \left(\frac{s_{\text{SI}}}{\text{Abs}}\right)^2_{\text{detector}} \quad (7)$$

Some comments should be made about Equation 7. Uncertainty generated during injection, flowing through tubing, and so on, is independent of the light absorbed (or transmitted) by the sample; therefore, this term should obey Equation 2. The “detector” term in Equation 7 is a typical shot-noise term and will obey Equation 3. With  $k_2$  calculated previously for the diode-array detector in the case of ferroin,  $k_1$  (Table 1) can be deduced from Equation 7 by nonlinear regression analysis (Table 2).

The plots in Figure 1 give much relevance to the relative uncertainty for low absorbances. A different perspective can be obtained when the calculated values for uncertainty constants (calculated from Figure 1-type plots) are used to simulate how the absolute uncertainty changes with absorbance. In this case, much more relevance is given to high absorbances. This makes it possible to check the agreement among experimental points and simulated lines. With  $k_1$  and  $k_2$  values as found above, the simulated behavior of the combined standard deviation can be given (Figure 3); it shows good agreement with the experimental points.

Because  $k_1$  and  $k_2$  are similar, the uncertainty introduced by the flow system cannot be neglected, and because  $k_3$  (the effect of cell positioning) was not relevant in the case of batch samples, no improvement of precision (limit of detection, etc.) should be expected just when an SI device is used. Similar conclusions may apply to flow-injection systems.

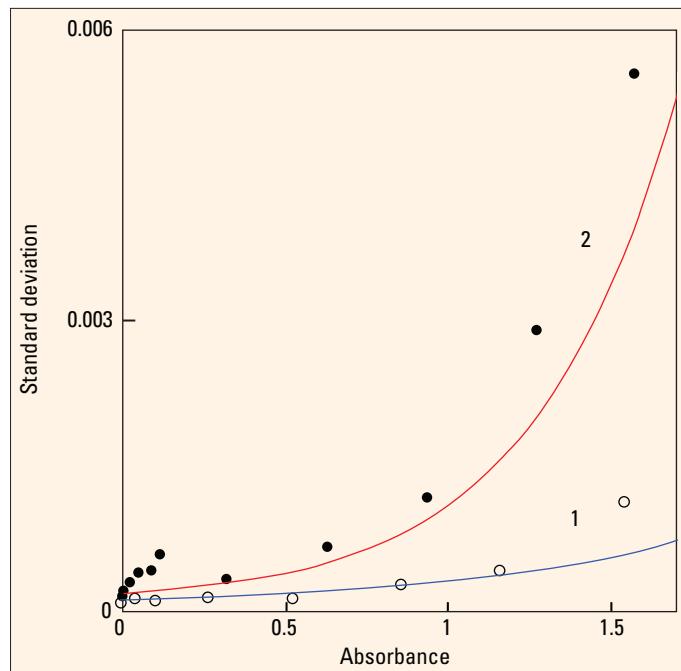


FIGURE 3. SI system performance.

Open circles indicate detector uncertainty; filled circles indicate uncertainty for the whole SI system. Line 1, simulated behavior of shot noise; line 2, simulation of the combined effect of shot noise and cell positioning.

## Conclusions

The spectrophotometric uncertainty associated with shot noise and cell positioning has strongly decreased during the past 30 years because of improvements in instrumentation and cell-holder designs. In general, no significant uncertainty is introduced by cell positioning when the cell holder is in good condition and the cell fits tightly in it. In the low-absorbance region, the condition of the cell holder is of prime importance in obtaining low uncertainties and has a great impact on the detection limit. Diode-array spectrophotometers are more precise, whereas for photomultiplier instruments, uncertainty is higher in the low-absorbance region. In flow systems, the injection step may contribute significantly to the uncertainty, which should be taken into account.

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