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The quantitation of fluorescence by photography

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ABSTRACT

A method based on theory has been developed for the photographic quantitation of fluorescent substances. DNA stained with ethidium in agarose gels is used as an example of an application of this method. In the course of developing this method we have demonstrated that the empirical methods employed by other authors can give rise to large systematic errors. We have also developed an approximate method based on photographic theory, avoiding the use of digital integration which is required by the rigorous method.

INTRODUCTION

Detection methods based upon fluorescence are widely used in the chemical sciences because they offer sensitivities several orders of magnitude higher than can be obtained by other optical techniques. Of particular current importance is the use of enhanced ethidium fluorescence (1) for the detection of nanogram quantities of nucleic acids after gel electrophoresis.

Photography, which provides the simplest method of recording such data, suffers from a serious deficiency in not permitting simple quantitation of the species present. We describe here the steps that can be taken in order to achieve accurate quantitation from fluorescence photographs. Furthermore we show that two of the empirical procedures that have been employed by other authors (2, 3, 4, 5) can give only approximate values since they fail to account for the logarithmic nature of the photographic process.

The photographic characteristic curve, a plot of the optical density of the developed film against the logarithm of the exposure, (Figure 1) may be considered to have three domains:

A. Underexposure: In this region the curve turns sharply upwards with increasing exposure.

B. Correct exposure: In this region there is a linear relationship between the optical density of the developed film and the logarithm of the exposure. The slope of this region of the curve is denoted by γ, which is the index of contrast of the photograph.

C. Overexposure: In this region further increments in exposure lead to decreasing increments in the optical density of the developed film.

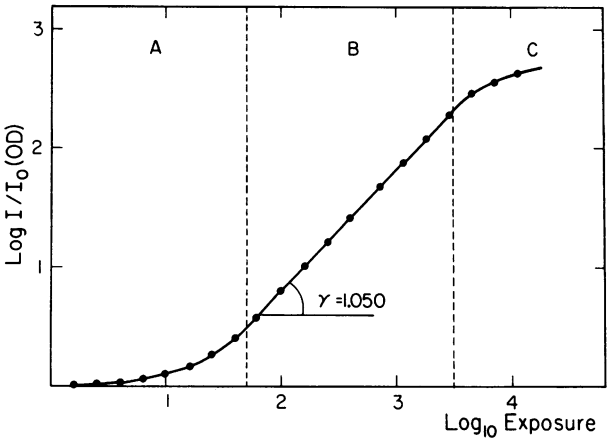

FIGURE 1. The characteristic curve of Kodak Plus-X film measured from a microdensitometer tracing of the image of a calibrated step wedge. Development was for 5' in Kodak D11 at 20°C.

From a consideration of Figure 1, it is clear that photographic quantitation of fluorescence intensity can be conveniently achieved only if both the background absorbance of the film and the maximum absorbance in the most intense peak fall within the linear region of the curve (Figure 1B). Since numerous factors can affect the precise shape of the characteristic curve, it is necessary to evaluate this curve whenever quantitation of fluorescence by photography is attempted. It is important to emphasize that both axes of the curve are logarithmic, necessitating the introduction of an exponent when relating the optical density of the developed film to the intensity (J) of the fluorescence (Equation 1).

$$J \propto 10^{D/\gamma} - 1 \qquad 1$$

D is the optical density of the developed film above background.

Under ideal conditions the intensity of the fluorescence is directly proportional to the quantity of the fluorescing species. For a band on an electrophoresis gel, where the concentration of a species is not uniform across the width of the band, it is necessary to integrate Equation 1 nu-

merically over the width of the band in order to determine the total quantity (Q) of the fluorescing species (Equation 2).

$$Q \propto \int_{band} J dx \approx \sum_{band} (10^{D/\gamma} - 1)\, \Delta x \qquad 2$$

Digital integration of fluorescence intensity by the procedure described above has been used in previous work (6, 7) to quantitate DNA in electrophoresis gels after staining with ethidium.

By comparison with fluorescent species, the photographic quantitation of absorbing species (e.g. in electrophoresis gels stained with an absorbing dye such as Coomassie Blue) is straightforward since it does not require the introduction of the exponential term $10^{D/\gamma}$. This follows from the fact that the intensity of the transmitted light (I) is logarithmically related to the amount of the absorbing species in the light path by the Beer-Lambert Law

$$\log_{10} (I_o/I) = \epsilon\, c l \qquad 3$$

where c is the concentration, l is the path length, ϵ is the extinction coefficient and I_o is the intensity of the incident light. Since within the linear range, the optical density of the developed film is proportional to the logarithm of the exposure (see Figure 1) the amount of absorbing material is directly proportional to the difference (E) between the background absorbance and the absorbance within the image of the absorbing species. Peak area measurement is therefore the correct method of quantitation (Equation 4).

$$P \propto \int_{band} E dx \approx \sum_{band} E \Delta x \qquad 4$$

P is the total quantity of the absorbing species in the band.

EXPERIMENTAL

DNA Restriction Fragments

30 μg PM2 DNA (gift of R. Watson) were digested with 30 units of HindIII restriction endonuclease (New England BioLabs) at 45°C for 4 hours. Partial digestion products were not observed to be present in the sample.

Electrophoresis

A vertical slab gel electrophoresis apparatus (Aquebogue) was used. Gels (4 mm thick) contained 1% agarose (Sea Kem) in 40 mM Tris-acetate pH 7.8, 5 mM sodium acetate, 0.1 mM EDTA, 1 μg/ml ethidium bromide (8).

Prestaining of the electrophoresis gels is necessary when very small restriction fragments are present since these may be partially

eluted during staining after electrophoresis. Electrophoresis gels containing high molecular weight DNAs can be stained overnight in 10 mM Tris-HCl, 1 mM EDTA pH 8, 1-2 μg/ml ethidium bromide. The concentrations of ethidium used for quantitative photography are high so as to ensure that the background fluorescence results in photographs that are within the linear range.

Photography

Gels and step density wedges (Kodak #2) were illuminated from below using short wavelength ultraviolet light from a transilluminator (Ultra-Violet Products, Inc.).

A 6" x 6" x 1/4" quartz plate (Amersil T08) was placed between the transilluminator and the electrophoresis gel in order to minimize local variations in illumination. The step density wedge was placed over a 1" x 6" x 1/8" strip of orange fluorescent plexiglass, lightly sandblasted on one side and taped at the edges to minimize stray light. This evenly illuminates the step wedge with red light.

The step density wedge was calibrated on an absolute scale using a Syntex AD-1 microdensitometer. The Syntex calibration disagreed by 25% with a calibration previously determined by Kodak, but agreed closely with an independent calibration performed by placing the wedge in the light path of a Gilford spectrophotometer.

Photographs were taken on 4" x 5" Kodak Plus X or Ilford FP4 sheet film through a Wratten 23-A filter. Development was for 5' at 20°C in Kodak D11 with continuous agitation.

Evaluation

Photographs were traced on a Joyce-Loebl microdensitometer. The characteristic curve of each film was determined by measurement of the step heights on the trace of the calibrated step wedge (see Figure 3b). If either the background absorbance or the maximum peak absorbance fell outside the linear range of the film response, the film was not further evaluated. Traces of correctly exposed photographs were digitized on a Hewlett Packard 9864A digitizer platen and evaluated on a Hewlett Packard 9820A calculator (Figures 2, 3).

RESULTS

For the purpose of illustrating the quantitative procedures employed we have used the HindIII fragments of PM2. The molecular weights of the seven fragments determined by a subtractive procedure (9) are listed in Table 1 (see also 10).

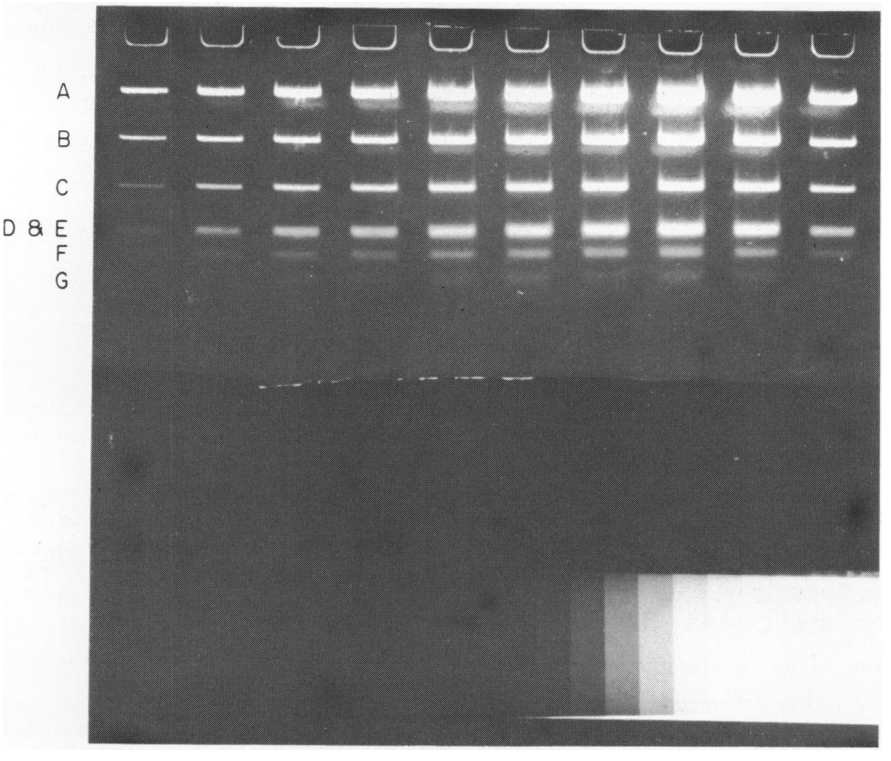

FIGURE 2. A quantitative photograph showing the resolution of species present in PM2 HindIII digests. Electrophoresis was for 2 hours at 2v/cm in a 1% agarose gel containing 40 mM Tris-acetate, 5 mM sodium acetate, 0.1 mM EDTA, 1 μg/ml ethidium bromide. Under these conditions fragments D and E migrate as a single species. In some places the images appear double because of reflections from the lower surface of the quartz plate. This did not interfere with the quantitation procedures.

Since fragment A is large compared to all of the other fragments it cannot be accurately quantitated in the same electrophoresis channel as the smaller fragments (see Figure 3a). It has therefore been excluded from this discussion. Deliberate use has been made of the small difference in mobility between fragments D and E to obtain photographs in which these species are not resolved.

The relative masses of the fragments B through G were determined from two independent sets of traces by the integrated transform procedure outlined in the introduction. These are listed in Table 2, column 1. Excellent correlation with the relative masses determined subtractively (Table 1, column 2) can be seen to hold for all but the smallest fragments, where the errors in both methods are maximal. The apparent relative

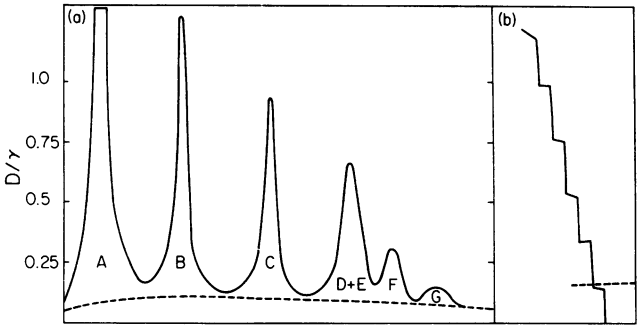

FIGURE 3(a). Microdensitometer tracing of a channel from the photograph shown in Figure 2. The vertical scale has been expressed in units of D/γ. For the purpose of quantitation, the background absorbance (---) was subtracted as indicated.

FIGURE 3(b). Microdensitometer tracing of the image of the wedge shown in Figure 2. The non-flat appearance of the steps is due to minor variations in the intensity of illumination of the wedge. Vertical step heights are measured at the edges of each step. Each step on this image represents 0.20 optical density units on the calibrated wedge, and is therefore equivalent to 0.2 D/γ units on the developed film.

TABLE 1. Molecular Weights of PM2 HindIII Fragments

Fragment	MW x 10^{-6} daltons	Fraction of (Total - Fragment A)
A	3.5	--
B	1.34	0.502 ± 2%
C	0.61	0.222 ± 5%
D	0.34)	0.194 ± 5%
E	0.27)	
F	0.15	0.057 ± 20%
G	0.06	0.025 ± 50%

masses were also determined by measuring peak heights and by area integration. The results of these measurements are listed in Table 2, columns 2 and 3 respectively. In each case the results obtained by these measurements differ significantly from the results obtained by the integrated transform method. Peak area, which at first sight would appear to be the more suitable measurement, actually shows greater errors overall. Peak height measurement suffers from the obvious defect of neglecting the effects of band shape. In the present example the use of peak height measurement could lead one to the incorrect conclusion that

TABLE 2. Photographically Determined Relative Mass of PM2 HindIII Fragments

Fragment	1* $\Sigma(10^{D/\gamma}-1)\,\Delta x$ band	2* Height	3* Area
B	0.514 ± 3.4%	0.430 ± 8.8%	0.386 ± 5.2%
C	0.227 ± 5.6%	0.278 ± 2.7%	0.246 ± 8.8%
D and E	0.192 ± 4.3%	0.192 ± 8.9%	0.250 ± 6.1%
F	0.049 ± 7.4%	0.076 ± 14%	0.084 ± 11.9%
G	0.017 ± 20.3%	0.024 ± 18.7%	0.034 ± 16.3%

*Averages and standard deviations are based on 10 measurements of separate channels in 2 electrophoresis gels.

the peak containing fragments D and E, in fact, contained a single species. The standard deviations on the peak height and peak area measurements are significantly higher than those on the integrated transform. This results from the systematic effects of sample loading on the apparent relative masses determined by either peak height or peak area measurements.

The Parabolic Approximation

Peak area and peak height are shown to be unsuitable measurements for any but the most approximate estimates of the relative concentrations of species in a resolved mixture. Since digital integration facilities are not universally available, we have developed an alternative approximate method based upon the assumption that the image of a peak has a parabolic height to width relationship (see Figure 4).

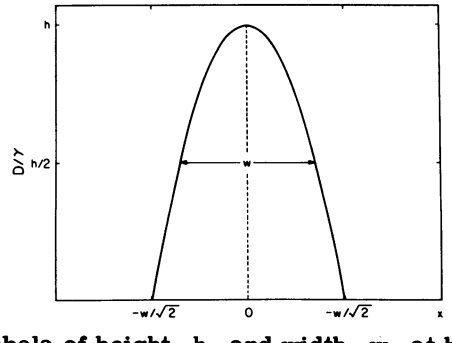

FIGURE 4. A parabola of height, h, and width, w, at half height is taken to represent the vertical height/width relationship of a band image for the purposes of approximation. The main deviation from this relationship usually occurs in the tails of a band profile which make only a small contribution to the integral.

Calibration of the photograph remains an essential feature of this method since it is necessary to show that both the baseline absorbance and the maximum peak absorbance lie within the linear range of the film response. Calibration is also necessary for measurement of the peak height, h, which must be expressed in units of D/γ. The width of the peak, w, is measured at half height and is in arbitrary units. The transform of the parabola has the form

$$I = \int_{-w/\sqrt{2}}^{w/\sqrt{2}} \{ 10^{(h - 2h x^2/w^2)} - 1 \} \, dx \qquad 5$$

This integral (I) has been calculated for values of $0 < h \leq 1$ (this corresponds to the useful range of the linear regions for most photographic materials) and has been shown to closely fit the polynomial (Equation 6) over this range.

$$I = w (2.28 h + 0.93 h^2 + 3.327 h^3) \qquad 6$$

Equation 6 has been evaluated for each of the PM2 HindIII fragments from photographs such as that shown in Figure 2. The normalized values for fragments B through G are listed in Table 3.

TABLE 3. Estimates of Relative Mass of PM2 HindIII Fragments by Parabolic Approximation

Fragment	$w(2.28 h + 0.93 h^2 + 3.327 h^3)$
B	0.498 ± 6.9%
C	0.223 ± 7.3%
D and E	0.206 ± 8.2%
F	0.053 ± 8.7%
G	0.019 ± 15.4%

Comparison of these values with those in Table 2, column 1 shows excellent agreement for all of the fragments. The larger standard deviations on the individual measurements are due primarily to random errors associated with peak width measurements.

DISCUSSION

It has been shown that proper evaluation of fluorescence photographs can give accurate values for the relative concentrations of the fluorescent species. However, the full procedure as outlined here does require digital integration and therefore, will prove inconvenient for many laboratories wishing to make use of fluorescence photographs for quantitation. The parabolic approximation has been developed to overcome the

need for digital integration. It provides a more suitable measurement than either peak area or peak height since full consideration is given to the logarithmic nature of the photographic response. It remains an approximation however, and as such should be applied with caution when the peak profile deviates markedly from that of a parabola.

Additional factors that must be considered when quantitating fluorescence photographs are: 1) The concentration of the fluorescing species must be sufficiently low to avoid attenuation of the exciting light. This is a potential problem when weakly fluorescent species are being estimated. 2) Differing affinities for a fluorescent stain, and differing quantum efficiencies of the fluorophor must be taken into account when estimating the relative amounts of diverse species. This is not a significant problem when estimating the amount of linear double stranded DNA by ethidium fluorescence, but becomes a potential problem when single stranded or synthetic polynucleotides are being quantitated (11).

Because of the strong effect of superhelix density on the binding of ethidium to closed circular DNA, there is a potential problem when estimating the relative amounts of closed circular DNAs. We have overcome this difficulty in previous work by photolytic nicking of the closed circular DNA in stained gels by exposure to high intensity ultraviolet light for 1-2 minutes prior to restaining and quantitative photography (6, 7).

Some attention should be paid to the factors that affect the suitability of a photograph for quantitation. High contrast photographs (large values of γ) will generally give more accurate values than low contrast photographs (low values of γ). This consideration, together with generally irreproducible results led us to abandon Polaroid negative films for quantitative use. Maximum contrast is obtained upon prolonged development of medium speed films in vigorous developers such as D11. Other factors affecting contrast include the film type, storage conditions, development temperature and exposure time (reciprocity failure). If format sizes smaller than 4" x 5" are to be used, attention must be paid to the contribution of adjacency effects that occur when the dimensions of the image are small. Such effects are minimized by prolonged development with continuous agitation.

The considerations discussed in this work apply equally to fluorography where attempts have been made to quantitate by peak area measurement (12). Prefogging of the film used by these authors has the effect of bringing the background into the linear region of the characteristic curve,

but does not overcome the need for exponential integration. At the present time we do not see any convenient method for measuring the characteristic curve of a fluorographic image since exposure time plays an important role in determining this function. In the absence of such a measurement, the most accurate method of quantitation must still be excision of the radioactive species followed by direct counting.

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