An Instrument for Digital Matrix Photometry

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A new instrument was conceived and designed for quantitative measurement of chromophoric areas or colored spots such as are produced in (e.g.) thin-layer chromatography. The areas to be measured are subdivided grid-like into small subunits, and the absorbance of each of these is measured. The sum of absorbances for all subunits is directly proportional to the total amount of light-absorbing substances in a spot. The absorbances of the subunits are measured with a photodetector that contains hundreds of microscopically small photodiodes, arranged in a precise geometric array. The photodiode array is interfaced with a computer via an analog-to-digital converter for numerically integrating the individual signals from each photodiode. With this analytical system, quantitation of light-absorbing substance is accurate and precise for areas of different sizes, shapes, and internal irregularity.

Additional Keyphrases: quantitating analytes in spots of various sizes and shapes in a planar support medium • analytical systems • new instrumentation • numerical integration by solid-state photodiode array • electrophoresis • thin-layer chromatography • densitometry • immunodiffusion • data acquisition

The accurate quantitative determination of the amount of an analyte irregularly distributed in spots of varying sizes and shapes is one of the largely unresolved problems in chemical analysis, even though many analytical methods—e.g., the various forms of electrophoresis, thin-layer chromatography, immunodiffusion, thin-layer chemistry—depend on quantitation of some reaction product in a plane. Current densitometric or colorimetric methods for quantitation of such areas are at best only semiquantitative (1, 2).

It occurred to us that this problem of quantitation could be considered a form of image analysis, and that the necessary spatial resolution and digitization might be accomplished by use of a photodiode array and computer. Light from a lamp passes through filters and then to the chromophoric area on a planar support medium. Part of the light is absorbed by the chromophore, the rest passes through a narrow slit and falls on the photosensitive areas of the photodiode array. Idealized, if the light passing through the specimen is perfectly parallel, the light received by the photodiode should come only from areas of the specimen above that have the exact dimensions and linear arrangement of the individual photosensitive elements of the photodiode array. The voltage signal from each photodiode is input to the computer after analog-to-digital conversion. As the specimen is moved in discrete steps over the photodiode array the entire chromophoric area is scanned in the form of a digital matrix, the combined elements of which represent a digitized image of the chromatophore distribution. The chromophoric area is now completely described in the computer in digital matrix form. This digital matrix may be used as a data base for any mathematical transformation and for various forms of data output. Figure 1 illustrates the basic design of the instrument.

Materials and Methods

The Instrument

Components: Our analytical system is based on a solid-state photodiode array (RL-512-G; E.G. and G. Reticon Co., Sunnyvale, CA 94086). This detector is a linear array, 1.28 cm long, of 512 discrete photodiodes on 25-μm centers. Each diode has an associated storage capacitor, which integrates photocurrent. Associated amplifier and clocking circuit boards (RC 100/105, E.G. and G. Reticon) sequentially read out each of the 512 capacitors after a start pulse. Analog voltage output of each photodiode was converted to a 12-bit binary number by a high-speed analog-to-digital (A to D) converter with an 8-μs conversion time (no. ADC 1102; Analog Devices, Norwood, MA 02062).

For system control and data processing we used a Model 9835A desktop computer (Hewlett-Packard, Fort Collins, CO 80525). Digital output of the A to D converter was input to the computer in real time via direct memory access on a 16-bit interface cable (Hewlett-Packard, no. 98032A). All system-control and data-processing programs were written in Hewlett-Packard BASIC language. Computer-generated absorbance data were plotted on an X-Y plotter (Model DMP-4; Houston Instruments, Austin, TX 78753).

Digital logic circuits were designed to interface the computer, A to D converter, photodiode array circuitry, and mechanical drive. Most of the interfacing was designed with use of standard integrated circuits.

A micrometer drive was used to advance the chromophoric area over the photodiode array. The micrometer was driven by a stepping motor, controlled by the computer.

The light source for the optical system was a 120-V, 300-W, quartz halogen lamp (no. ELH; General Electric, Cleveland, OH 44112). Lamp voltage was controlled by a variable autotransformer (no. 3PH116B; Superior Electric, Bristol, CT 06010). Monochromatic light (510 nm) was obtained by placing a ground glass diffuser, an infrared blocking filter, and a three-cavity interference filter with a 10-nm bandpass (Dichromatic Optics, Hudson, MA 01749) between the lamp and the specimen plane. To optimize the resolution of the A to D converter, we adjusted the light output to obtain approximately 3600 A to D units between 0 and 100% transmittance. A light-tight instrument enclosure was machined from aluminum plate and black-anodized, to decrease light scatter. Air was circulated with a fan to prevent overheating by the illumination system.

Instrument operation: At the beginning of each experiment we calibrated the system by measuring the dark signals representing 0% transmittance and the blank signals representing 100% transmittance. This calibration procedure generates a dark-signal linear array [d] (Equation 1) and a blank signal linear array [b] (Equation 2).

\[ [d] = [d_1, d_2, \ldots, d_{512}] \]  

(1)
The specimen is stepped over the photodiode array by the mechanical drive under computer control (Figure 1). At position intervals determined by programming, the output of the photodiode array is transmitted to the computer, where the 512 signals are stored. As this process is repeated a signal matrix, \( [s_{ij}] \), is generated (Equation 3).

\[
[b_j] = [b_1, b_2, \ldots, b_{512}] 
\]

\[
[s_{ij}] = \begin{bmatrix} 
 s_{1,1}, s_{1,2}, \ldots, s_{1,n} \\
 s_{2,1}, s_{2,2}, \ldots, s_{2,n} \\
 \vdots \\
 s_{m,1}, s_{m,2}, \ldots, s_{m,n} 
\end{bmatrix}
\]  

Here, \( j \) ranges from 1 to 512 and represents the photodiode number in the array; \( i \) represents the row number. The distance between the rows and the number of rows is determined by the computer program. We chose 25 \( \mu \)m as the distance between rows.

The photometric data contained in the calibration arrays and the specimen signal matrix may be subjected to mathematical transforms of interest. We selected the conventional absorbance function to convert photometric values to chromophore concentration. Each element of the signal matrix was transformed to absorbance by use of the transformation of equation 4.

\[
A_{ij} = \log\left(\frac{[b_j] - d_j}{[s_{ij}] - d_j}\right) 
\]

The total quantity of chromophore, \( Q \), is obtained by summing \( A_{ij} \) over the entire signal matrix (Equation 5).

\[
Q = \sum_{ij} A_{ij} 
\]

Materials

Red dye. U.S. Certified Food Coloring, 25 g/L (Durkee, Cleveland, OH 44115).

Bovine serum albumin (Sigma Chemical, St. Louis, MO 63178). Albumin was dissolved in 9 g/L sodium chloride solution to give the following concentrations: 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 g/L.

Ponceau S stain. (Helena Laboratories, Beaumont, TX 77704). This stain contains, per liter, 5 g of Ponceau S, 35 g of sulfosalicylic acid, and 35 g of trichloroacetic acid.

Cellulose acetate sheets. Cellulose acetate (cat. no. 3023; Helena Laboratories).

Glass cuvette. A special glass cuvette was constructed by placing glass coverslips as spacers between the ends of two glass microscope slides. This created a plane-parallel space of 0.18 mm between the glass slides, and this space was used as a cuvette, which filled by capillary action. The filled cuvette, when placed horizontally over the photodiode array, provided a medium of uniform optical density.

Procedures

Preparation of chromophoric areas. To produce these areas we placed albumin solutions of various concentrations and volumes on cellulose acetate sheets and stained these according to reference 3. The cleared cellulose acetate sheets were removed from the Mylar backing and mounted between two glass cover slips.

Three-dimensional plots of the chromophoric areas. The information in the signal matrix was subjected to a viewing transformation and the resulting matrix was plotted on an X-Y plotter. Because of the high density of data in the signal matrix, only every 15th row was used in this transform. The resulting plots project the absorbance of the specimen as elevations above the x-y plane.

Results

Instrument Operating Characteristics

Stability. We tested the performance of representative single diodes, as well as that of the entire array. To test the stability of single photodiodes, we arbitrarily selected photodiodes number 1, 100, 200, 300, 400, and 500 in the photodiode array.

Dark signal. Sequential single measurements taken from these single photodiodes at the sampling interval of 40 ms varied up to 6 A to D units of the full scale of approximately 3600 A to D units. When we used signal averaging of multiple (up to 10) readings, the dark signal varied by only 2 A to D units after 15 min of instrument warm up.

Blank signal. After 15 min of instrument warm up, the blank signal from these photodiodes varied by about 40 A to D units, or 1.1% of full scale. The stability of the blank signal significantly improved when separate photodiodes at each end of the array were used as reference photodiodes. The ratios between the blank signal from the test and the reference photodiodes varied by less than 0.6%.

Photometric precision. The variation of the signals between the 512 individual photodiodes on single scans of the photodiode array were tested. For these experiments the glass cuvette was filled with red dye solution in various concentrations so that absorbances from 0.05 to 2.0 were obtained. To test for inter-photodiode variation, we made single-array scans of each of the dye solutions. The coefficient of variation of the readings in the absorbance range from 0.4 to 1.1 absorbance was 0.8%. Below and above this range the CV values were 1.7 and 2.6%.

The variation of the signals from scan to scan was also determined with these same red dye solutions. For this purpose the absorbances of the 512 photodiodes were summed for each scan. In the absorbance range from 0.4 to 1.1 the CV values were 0.16 to 0.25%, while below and above this range the CV values were 1.6 and 1.0%.

Photometric linearity. A series of dilutions of a stock solution of red dye of known concentration was prepared and the absorbances were measured, taking the average of eight array scans. Regression analysis of the data showed that absorbance \( y \) was a linear function of concentration \( x \) up to absorbances of 1.2 \( (n = 7; y = 0.9159x - 0.0060; r = 0.9998) \).

Quantitation of irregular Chromophoric Areas

Variation in sample volume with fixed albumin concentration. In this experiment the chromophoric areas were produced as described under Materials and Methods with
0.5-5.0 μL samples of an 6.0 g/L albumin solution. These areas were scanned as described under systems operation and the quantity of chromophore was determined according to equation 5. The absorbance sums (y) were linearly related to sample volume x (n = 6; y = 11.1x + 0.867, r = 0.9987).

The three-dimensional plots (see under Methods) of the six chromophoric areas used to obtain these six data parts are shown in Figure 2 (A-F), and clearly demonstrate the irregular distribution of the chromophore in the specimen plane.

Variation in protein concentration with fixed sample volume. A series of albumin solutions—3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 g/L—were prepared and 1.0 μL of each of these placed on the cellulose acetate sheets, stained, and scanned as above. There was a linear relation between the sum of absorbances of the chromophoric areas (y) and protein concentration (x) (n = 6; y = 1.63x + 0.356, r = 0.988).

Markedly irregular chromophoric areas produced with samples of fixed protein concentration and volume. To produce areas of markedly irregular distribution of the chromophore, we placed 2.0-μL samples of the 6.0 g/L albumin solution on the cellulose acetate sheets in a deliberately irregular fashion. The resulting spots were scanned as above. The lowest and highest values of the summed absorbances of these four spots differed only by 3.4% (Figure 3 A-D).

Discussion

Many techniques in analytical chemistry are based on the production of irregular distributions of a chromophore or other light-absorbing materials in a planar support medium, such as thin-layer chromatography, electrophoresis, immunodiffusion, etc. The applications of these techniques have been qualitative or semiquantitative at best. Adequate quantitative analysis has been achieved in a number of forms of electrophoresis where the resulting light-absorbing patterns approximate homogeneous rectangles. Photometric errors are substantial if the chromophoric area is not homogeneous or if the scanning slit extends beyond the chromophoric areas. However, the general problem of quantitation of a light-absorbing substance irregularly distributed in a planar support medium has not been resolved satisfactorily up to this time.

The fundamental problem of quantitative analysis in thin-layer media is one of numerical integration. Goldman and Goodall (4) and subsequently Pollak and Boulton (5) described instruments for the quantitative analysis of thin-layer chromophoric patterns in which, for the purposes of numerical integration, the area to be scanned was mechanically moved over a small spot of light. These workers recognized that the process of numerical integration was highly data intensive and well suited for computer analysis. These studies confirmed the effectiveness of the numerical integration for performing quantitative analyses.

Our system is a new instrumental approach to this problem of quantitation in which numerical integration is accomplished by a solid-state photodiode array (6). Originally developed for optical character reading and pattern recognition, photodiode arrays have been incorporated in spectrophotometers as multiple-wavelength detectors (7). Their reliability, geometric precision, and photometric accuracy have been well established in these applications. These same operating characteristics appear to make the photodiode array an ideal detector for digital matrix photometry. The very small size of the individual detector elements, their geometric arrangement and their electronic control obviate the need for complex and costly optomechanical components. The photodiode-array-based digital matrix photometer described here acquires a high-resolution two-dimensional photomatrix of chromophoric patterns. The photomatrix, in conjunction with dark and blank matrices, provides a complete description of the photometric information in a chromophoric pattern. Per square centimeter, the digital matrix in our system contains 160,000 data points; that of Goldman and Goodall contained only 400 (8). A basic requirement for the digital matrix analysis to be valid is that the areas represented by one data point must have, to a first approximation, a uniform distribution of chromophore. Any significant degree of nonuniformity will result in nonlinearity of absorbance signal. As a result, a high degree of irregularity requires a high degree of resolution.

There is a direct relationship between the integral of the absorbance of the total chromophoric area and the concentration of analyte, and, because of the high resolution, this relationship holds true over a wide range of irregularity of shapes and internal distribution of chromophore.

For the conceptualization of the system we have assumed ideal operating characteristics such as perfectly parallel light, absence of light diffusion and diffraction in the sample, and complete independence of the individual photodiodes from each other.

We will examine the influence of deviations from these idealized operating characteristics and the optimization of the density of data points for a given irregularity of the distribu-
tion of the chromophore in a subsequent paper. However, we have shown here that highly precise and accurate quantitative measurements of a chromophore can be made with our system in chromophoric areas of great irregularity.

References
3. Serum Protein Electrophoresis Procedure No. 1, Dec. 1977, Helena Laboratories, P.O. Box 752, Beaumont, TX 77704.