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Important design features of a system for the densitometric analysis of two-dimensional flat-bed separations

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ABSTRACT

Densitometric instruments for the analysis of one-dimensional separations employ almost universally electromechanical scanning systems. Electronic scanning has been almost universally adopted for two-dimensional work. This permits the incorporation of a number of important features which an electromechanical system cannot provide. Some of these features are specific for densitometric equipment and are not needed in other applications, and these features are discussed. Modern image processing theory was applied to densitometry and densitometry analysis of two-dimensional flat-bed separations with emphasis on electrophoresis. The described features and techniques were implemented and tested on an experimental system. Only the software aspects of that system are discussed here.

INTRODUCTION

Two-dimensional (2D) electrophoresis can provide extremely high separating powers and it is therefore mainly applied to the analysis of multi-component solutions [1]. Techniques for the examination and interpretation of the resulting separations are the focus of the initial part of this paper. Most of the discussion applies also to 2D thin-layer chromatography (TLC), although the techniques are little used in practice.

DENSITOMETRIC ASSESSMENT

Analysis of proteins, nucleotides and other complex biological substances is one of the most significant applications of 2D electrophoresis. A high-resolution electrophoretic separation may result in hundreds and even thousands of spots. Evaluation by visual inspection is then an extremely tedious and error-prone endeavour. Quantification manually with any degree of accuracy is almost impossible and computer analysis is required. The main aim of this paper is to discuss the most important features that such a system should exhibit and to describe an experimental system for implementing these features at reasonable cost.

Optical densitometry is the technique commonly used to acquire input data for the analysis. The scanning of 2D separations necessitates a number of

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features usually not found in instruments primarily designed for one-dimensional (1D) work [2]. The differences are discussed below.

The approach adopted is to regard a 2D separation as a picture and to utilize modern techniques of image processing [3]. This approach can be extended also to the analysis of 1D separations, but is of little advantage here, mainly because most current 1D densitometers employ "slit scanning" instead of "point scanning" [4].

The spatial resolution of a scanning system is determined by the number of measured data points (pixels) per unit image area. The resolution of most standard video scanners may not be sufficient for high-grade work and special designs may have to be used. Another drawback of most standard devices is an unequal resolution between the x- and y-directions. Very important is a linear grey scale of the sensor, the gamma factor of which should be unity. Gamma is the ratio of scanner output signal to the logarithm of light energy per unit area, differentially ($\gamma = \Delta u_{out}/\Delta \log E$).

Processing of the acquired input data is inevitably done on a computer, which mostly is an integral component of the analysis system. The most cost-effective solution is to use a modern microcomputer, the performance of which rivals that of mainframe computers of a decade ago at a fraction of the cost. The processing software has to be tailored to the machine employed, because programs are rarely transferrable from one type of machine to another.

IMAGE SENSORS

The most important part of an electronic picture acquisition system is the video camera and its main element, the image sensor. Semiconductor area sensors, mostly charge-coupled devices (CCDs; Fig. 1a), are currently the most cost-effective solution [5,6]. They are available with up to 1300 × 1000 pixels per device [7]. Such area sensors provide a linear resolution of up to 200 μm on a 200 mm × 200 mm object. When that is still insufficient, CCD line sensors (Fig. 1b) can be employed. They are available with more than 5000 elements in a row, yielding a linear resolution of better than 40 μm on an object plane of 200 mm × 200 mm (many suppliers, *e.g.*, Philips, Thomson–CSF, Toshiba). The resolution obtainable on smaller substrates is

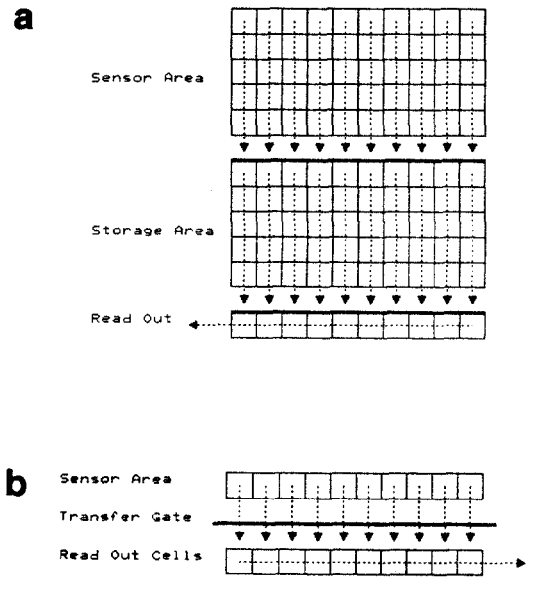

Fig. 1. (a) Disposition of a CCD area sensor with square sensing elements. During the exposure each element accumulates an amount of charge proportional to the intensity and time of illumination. The accumulated charges are usually transferred into a storage area and are then sequentially fed out line by line into an amplifier, where the charge is converted to a voltage signal. (b) Disposition of a CCD line sensor. At a given instant in time only one line of scanned image is sensed. When the charge packages are fed out, the sensor is displaced mechanically to scan the next line.

proportionally higher. Higher values than those quoted above are rarely warranted in this field. The main reason is the inevitable contamination of the background by small amounts of unwanted "garbage", which is difficult to distinguish from small spots of analysed material.

It should be noted that the practical resolution of a camera set-up is below the spatial distance of the pixels. The reason is that the modulation transfer function (MTF), a dynamic parameter, restricts the usable resolution to about 60% of the nominal value. A detailed explanation is beyond the scope here but can be found in the literature [8].

In some special cases, the available resolution can be increased by dividing the scanned image into segments which are acquired independently and then recombined in software. Another approach to raising the resolution that has proved viable is to scan the image, then to displace the sensor by half the distance between two of its neighbouring pixels

and to scan again. This displacement is usually performed by a simple piezo transducer which provides a small shift of half a pixel width with sufficient accuracy. However, the additional cost of these options is rarely warranted in systems for routine work.

When comparing line sensors with area sensors, cost and reliability are the main issues. Unless extremely high resolution is required, area sensors are generally preferable in both respects. The main advantages of line scanners, apart from their higher resolution, are simpler interfacing to the processing computer and a lower demand on its memory space. These advantages are generally more than outweighed by a significantly more complex mechanical and optical system.

MULTISPECTRAL SCANNING

Scanning at different wavelengths is an important aid for qualitative analysis. When CCD scanners are employed, the best approach to multi-spectral scanning is repeated exposure at different wavelengths of illumination. Filters or monochromators can be used to vary the sensed wavelength [2,9]. The additional time is negligible but the demand on memory space is considerable and increases the cost of the system.

The ease and speed with which spectral analysis can be carried out by electronic scanners promise to widen the field of applications of this technique. The spectral sensitivity of solid-state sensors ranges far into the infrared. This spectral range may become a useful extension of the currently used wavelengths in analysis.

LASER SCANNING

Still higher resolution than that which line sensors offer is rarely needed or even desirable. In the rare cases where still higher values are needed (*e.g.*, in some research applications), they are best achieved by laser scanning [10]. Here the scanning beam is deflected in both the x- and y-directions by mechanical or optical means. The deflecting mechanism must be built to very close tolerances, which increases the cost of the design. Also, the data acquisition time is very long. Another drawback is the fixed wavelength of all less expensive lasers. At low concentrations it is highly desirable that the operating wavelength of the densitometer coincides with the absorbance maximum of the investigated material. Laser scanners do not provide this option and are not suitable for spectral analysis.

UV SENSITIVITY

Silicon-based area sensors and line sensors are not very sensitive to UV radiation. However, sensitivity in the UV region is advantageous in many applications. Fundamental physical reasons have so far prevented the extension of the spectral sensitivity of CCD sensors into the UV region but an indirect method is possible by coating CCD sensors with a down-converting phosphor which shifts their range of sensitivity far into the UV region [11–13]. Unfortunately, the demand for UV-sensitive CCD sensors has not yet justified the large-scale production of phosphor-coated devices, which are therefore very expensive.

Another approach is the use of an image intensifier with an extended UV response in conjunction with CCD scanning. The sensitivity of the instrument is thereby significantly increased but at the price of a lower spatial resolution.

In the future, another option might be the use of pyroelectric sensors [14]. These are effectively heat sensors and their spectral sensitivity curve easily covers the whole range of wavelengths which are employed in the densitometric analysis of flat-bed separations. However, with the present state of technology the sensitivity and the spatial resolution of this type of sensor are inferior when compared with standard silicon-based CCD sensors.

OTHER HARDWARE CONSIDERATIONS

Most modern densitometers use a built-in computer as an integral part of the hardware. Often the machine serves for both the data acquisition and the subsequent analysis. An AT-class computer is mostly sufficient and leads to significant cost savings when compared with custom designs.

Many of the operations involved are fairly memory intensive. The machine selected should therefore have a sufficiently large memory bank and use a modern operating system that can handle the whole amount of memory directly. The software employed

must match the machine and its operating system. Portability of the software from one type of machine to another can rarely be assured. This caveat applies still more to specially designed hardware. Hard disks with a very large storage capacity (120 Mbyte or more) or in the extreme optical disks can be used as a data base, so that the results are available for future reference. Another quality criterion for large installations is the ability to link the complete system to an existing network such as an IEEE-488 net or higher speed links. All these devices are available at moderate cost.

PREPROCESSING

The processing operations can be divided into two functional stages. The first, the "preprocessing" stage, treats data from individual small areas as separate entities not related to data from other areas. Stage two then has the purpose of providing global information pertaining to the separation in its entirety. In many less sophisticated applications, preprocessing yields sufficient information. Only this stage will be considered here in depth. Final processing is too much application dependent to be covered in this paper.

The main operations carried out by the preprocessing stage are the following. The measured optical signals are made up of two components, one due to the optical response of the blank medium and the other to the response increment caused by the presence of separated material when a spot area is encountered. Only the latter component is useful, but its separation from the background is not always easy, because at larger concentration densities of the analyte the two components have a non-linear relationship. However, even when this non-linearity is small enough to be discounted, there are problems in isolating the response component due to the separated substance. However, the background response generally varies spatially in a random fashion, giving rise to what is called "background noise". For good accuracy and/or high sensitivity it is then necessary to take this variability into account. If the separated substance has a relatively narrow absorbance spectrum, two-wavelength (two-λ) scanning is probably the best technique for that purpose [15]. The method is described later. It is not very useful when the analyte has a broad absorbance spectrum, *e.g.*, in silver-stained protein separations. It may then be necessary to apply special techniques of signal processing.

Preprocessing can obviously be performed in many different ways, but the procedure described below seems to be both simple and efficient. Its structural organization is fairly typical although details of implementation may vary. It was adopted for the experimental system described below and performed in most respects equally well as and even better than some much more costly commercial systems used for comparison.

MEASURING MODES IN DENSITOMETRY

The first step in preprocessing is the segmentation of the picture into spotted and blank areas. High-frequency noise and other spurious signals have to be reduced to a minimum for a successful segmentation.

In the described system, a size threshold was employed to distinguish between contaminant noise and analyte signal. For partial elimination a two-step filter algorithm was used. It employs first a wide-band two-dimentional mask, then a narrow-band mask with median weighting. The mask sizes and the weighting laws were established empirically.

A special technique used fairly extensively in electrophoresis is autoradiography. Here it is also common not to examine the original separation but a photographic replica on transparent film. For quantitative analysis it is then necessary to take the gradation characteristics of the film into account.

A relatively new technique is photoacoustic spectroscopy. This holds some promise for special applications but has not yet found much acceptance in densitometry.

A detailed discussion of all these techniques and their limitations would exceed the scope of this paper. The interested reader is referred to the literature, *e.g.*, ref. 16.

DETERMINATION OF SPOTTED AREAS

A spot is defined as a contingent group of pixels which is spatially larger than an empirically chosen minimum. In the spot area the light attenuation exceeds the background attenuation $b_0(x,y)$ by at least a threshold ε. The implementation of these

simple relationships involves the solution of three by no means trivial tasks.

First it is necessary to find the mean value for $b_0(x,y)$, the attenuation of the blank medium. The procedure is given later. Pixels with attenuation values $b_s(x,y)$ more than ε above the mean value of $b_0(x,y)$ in the vicinity are judged to contain separated material if the criterion for a minimum number of contingent pixels is met.

SPOT BOUNDARIES

In principle, the contour is determined by the contingent sequence of pixels with response values which begin to increase from the background value $b_0(x,y)$ by ε or which begin to decrease by ε to $b_0(x,y)$:

$$b_s(x,y) \approx b_0(x,y) + \varepsilon \quad (1)$$

This simple approach does not lead to a reliable boundary detection in practice. Other popular methods involving the Laplace operator or direction-dependent masks are also unreliable. The main reason for the difficulties is the very shallow boundary zone without sharp transitions.

Therefore, another approach has been developed: the threshold ε is set to approximately twice the local Root Mean Square noise amplitude. Theoretical work [17] predicts that the boundary shape is approximately elliptical. An ellipse is then computed, meeting two conditions: the area of the ellipse must be equal to the area of all pixels with analyte in that cluster and the number of pixels that do not belong to the cluster must reach its minimum inside the ellipse. The synthetic contour is then overlayed on the screen for visual inspection (Fig. 2). More precise methods of contour estimation have been tested but the method described above has proven to be sufficient and the simplest to implement.

Theory also predicts an approximately Gaussian distribution of the concentration of separated material within a spot [17]. Provided that the noise floor and accordingly the threshold level ε are low, the loss due to pixels that are below ε does not significantly degrade the quantitative accuracy.

NOISE CONSIDERATIONS

The total noise components in a recording come from various uncorrelated sources. Usually the background noise of the blank medium contributes most significantly to the total noise and other sources of noise may then not have to be taken into account.

The next important noise mechanism is electronic noise from the sensor. The finite step size of the digitizer often presents an ultimate barrier if the sensor noise is kept to a minimum.

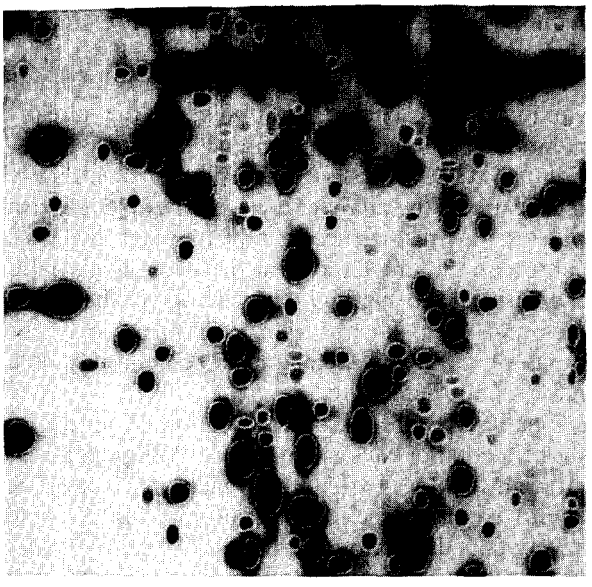

Fig. 2. Contours obtained after processing a 2D separation of cell proteins. Shown is approximately one quarter of the total picture area. Dots indicate the computed centre of gravity positions.

NOISE REDUCTION MEASURES

Low-pass filtering is a universal method and was consequently incorporated into the test system. Two stages of filtering are used, both of them employing two-dimensional masks. The first filter algorithm, with a fairly broad band pass, is mainly intended to reduce high frequency electronic noise. The second mask, with a much narrower band pass, serves for the partial elimination of background noise and other low-frequency components.

Another important feature of the system is the ability to scan the same separation repeatedly with subsequent frame averaging. This can be seen as a low-pass filter in the time domain and will only

reduce noise components that are uncorrelated in time and not those which are random in space.

The most effective method for the nearly complete elimination of background noise is the already mentioned two-lambda technique. Here the separation is scanned first at the spectral absorbance maximum of the analyte, then at a wavelength just outside its absorbance band. A pixel by pixel subtraction of the data from the two passes yields the result. The method only works if the blank medium has a flat absorbance spectrum over the range of the two wavelengths. The computational effort is not demanding.

The test system provides still another option for the reduction of background noise. Although not as efficient as the two-lambda method, it can be employed where the latter is not applicable, *e.g.* in silver-stained protein separations. The method relies on the fact that the spatial change of $b_0(x,y)$ is generally small so that for each pixel within the contour line b_0 can be approximated by linear interpolation between the values just outside the contour on both sides of the spot. For moderate demands on accuracy, the procedure can be further simplified by using the averaged value:

$$\overline{b_0(x,y_i)} \approx \tfrac{1}{2}[b_0(x_l,y_i) + b_0(x_r,y_i)] \qquad (2)$$

for all pixels on the same scan line within the spot contour, where i designates the sequential number of the respective scan line and x_l and x_r are the coordinates of the pixel on that line, which border the contour of the examined spot to the left and right, respectively.

FEATURE BASIS FOR QUALITATIVE ANALYSIS

Qualitative analysis relies to a large extent on the geometry of the spot distribution. The proposed system uses the coordinates of the centre of gravity of each spot as the characteristic feature. The determination of these parameters from the measured distribution of concentration $c(x,y)$ is computationally easy because very high accuracy is rarely needed. The quoted theory predicts an elliptical shape of the contour with the centre of gravity at the point where $c(x,y)$ and $b_s(x,y)$ reach a maximum. That maximum is generally flat and difficult to determine with any degree of accuracy. The problem can be solved with little error by replacing the coordinates of the maximum with those of the geometrical centre of the ellipse. For visual verification the centre in the experimental system is indicated on the display by a white spot. The coordinates of x_m and y_m together with the number of pixels enclosed by the contour are then considered as the characteristic set of data for each spot and compared with corresponding data from separations with known composition; x_m, y_m and the pixel counts for the individual spots are stored in long-term memory for later reference.

CORRECTION FOR GEOMETRIC DISTORTION

Evaluation of 2D separations relies very much on comparison of the examined separation with a standard or a template [18]. For this approach to provide useful results, the compared images must meet a well defined geometric correspondence. However, for reasons inherent in the separation process, the relationship between the coordinate system of individual scans can be complicated, *e.g.*, in the case of non-uniform shrinkage in the drying process. Special marker substances may be added if a substance does not contain sufficient prominent and easily identifiable components from which the geometric distortion can be computed. Constant scale differences between coordinate grids require only three markers; non-linear cases might require more markers. In the case of scale shifts the software in the test system corrects the position of the spots after the marker shifts have been identified.

QUANTIFICATION

It has to be considered that the relationship between optical response and concentration $c(x,y)$ is usually highly non-linear. Inherent linearity can only be assumed at very small values of the absorbance value $\alpha c(x,y)$, where α is the absorbance (sometimes called the extinction coefficient). Regardless of the magnitude of $\alpha c(x,y)$, a linear relationship between response and concentration can be expected for fluorescence measurements and radioassays, but for the latter only if direct measurement of the radiation is possible, which is rarely the case.

The total amount of substance in a spot is obtained by summation of $c(x,y)$ over all pixels

inside the contour. It can be seen that for a non-linear relationship between $b_s(x,y)$ and $c(x,y)$,

$$\int_A b_s(x,y) \neq \int c(x,y) \tag{3}$$

where A denotes the area for integration. It is thus evident that before summation a transform must be carried out that results in a linear relationship between the transformed response signal and the concentration.

The subject of linearization is extensively covered in the literature and a number of different transform rules have been proposed. The transform operations suggested in ref. 19 were incorporated in the software of the described test system.

For nearly transparent media such as electrophoretic gels, measurement of the optical transmittance is indicated and a logarithmic transform is then an adequate linearization operation. With strongly scattering media such as paper, transmittance is not practical and reflectance is used, and the linearizing operations are then much more involved.

STABILIZATION OF THE INTENSITY OF ILLUMINATION

Technically, the intensity of the illumination can be stabilized in different ways. The range of wavelengths required in our field is wide and therefore many densitometers are equipped with multiple light sources, each requiring an individual method of stabilization.

The system described here bypasses this difficulty by compensating for changes in the illumination intensity in the software. The intensity is measured by a separate light sensor and the output of that sensor is used by the software as a corrective scale factor. However, rapid changes in intensity cannot be taken care of in the software so that, *e.g.*, the power supply for the illumination should be a d.c. low-noise regulated source.

STREAK REMOVAL AND SEPARATION OF PARTIAL OVERLAP

Streaks can seriously affect machine reading but are not always avoidable. Destaining or similar measures can only alleviate the problem. Therefore, it is necessary to handle the effect of streaks in the preprocessing stage [20]. The elimination of partial overlap between spots uses similar software methods. Both tasks are carried out in a two-step approach outlined below.

The boundary of a cluster of pixels is checked for symmetry and size. If any asymmetry exceeds a certain limit or if the cluster is excessively large, the program assumes an anomaly and activates a subroutine. The gradient of $b_s(x,y)$ in the direction of the maximum asymmetry is computed. If the gradient is much smaller than could be expected from a Gaussian distribution, a streak is anticipated. Now the already mentioned elliptical contour is computed, but this time only taking into account areas of the spot that show normal gradients, thus excluding the streak direction. The method works well provided that the streak does not cover too much of the true spot area.

Overlap is detected by a similar test. An overlap is anticipated if the sign of the gradient in the direction of maximum asymmetry reverses and a saddle point is thus detected. Separation of overlapping spots was originally carried out by complementing the contour to both sides of the line of sign reversal to an elliptical shape. A different approach has recently been tested that additionally is capable of handling the overlap of more than two spots simultaneously. The technique employed here is erosion with subsequent dilation [21]. Erosion is normally performed independently of direction. However, there are indications that erosion enhancement in the direction of the asymmetry might be more efficient. Preliminary results are promising but a definitive conclusion has not yet been reached.

ENHANCEMENT OF DISPLAY FEATURES

Visual inspection of the scan is often the final step of the analysis. In that case feature enhancement by image processing can greatly simplify the visual examination.

A frequently employed technique for that purpose is edge enhancement. Here this method is not very useful because the software already creates a synthetic contour which is superimposed on the original contour.

Another very helpful tool is grey level transformation. Gradients in the image are artificially increased

so that the recognition of structures becomes easier. Grey levels can also be transformed into colour information as is known from satellite pictures. However, the user of such techniques must have a firm understanding of the effects of image processing. There is an extensive literature on the topic of image processing [22–27]. Pattern recognition principles can also be advantageously applied, although they are mostly targeted at applications other than densitometry [28–32].

FINAL PROCESSING

The so-far discussed preprocessing of the acquired densitometric data remains more or less unchanged regardless of the final purpose of the analysis. Final processing serves to evaluate the more global features of the acquired densitometric picture. Since the variety of tasks is great, only one of the final evaluation steps will be mentioned here. This is concerned with the comparison of the global structure of a scan with that of another one serving as a template.

The variant adopted here condenses in principle the information content of a separation into an ordered list of numerical values. The number of entries in that list is equal to the number of detected spots. However, that list can become too long to be handled efficiently, especially for biological samples. Interpretation can be facilitated by the fact that each entry consists of three values and thus can be represented by a vector in three-dimensional space. Vectorial addition of the individual entries yields a vector in multi-dimensional space which can be compared with or subtracted from the corresponding vector of a comparison separation by established mathematical procedures.

Sometimes only a fraction of the separation needs to be compared. Here the vectorial approach can also be useful, although a direct spot-by-spot comparison is then generally preferable.

TEMPLATE SELECTION

Frequently, the available advance information about the provenance of the examined sample is insufficient to select the most suitable template. It may then be necessary to investigate quickly a sometimes fairly large set of separations from storage. The vectorial approach can advantageously be employed. Separations which are to serve as templates are scanned and digitized only once. The acquired data together with the results of preprocessing are then put into library storage (mostly on hard magnetic disk) from where they are recalled when needed. Separations which are not intended to be used as templates are usually not stored in their entirely in order to limit the storage space. Storing the feature vector alone is generally adequate, more economical and facilitates search operations for retrieval. It must be considered that magnetic storage is subject to fading, and is therefore not suitable for the preservation of archival material. Optical disks offer much larger storage capacities and are not subject to fading [33].

CONCLUSIONS

The features discussed were built into an experimental system which was then used in electrophoretic separations. Emphasis was placed on a cost-effective and user-friendly realization. With very few exceptions the hardware was assembled from commercial off-the-shelf equipment around a standard AT-class computer. Only the illumination system had to be designed in detail. Therefore, a discussion of hardware details did not seem appropriate here. The software is the only aspect that was completely custom designed, but special attention was given to the possibility of using in a future version integrated mathematical packages which are not necessarily intended for densitometric use.

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