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AN EXPERIMENTAL HIGH-PERFORMANCE PHOTODENSITOMETER FOR QUANTITATIVE CHROMATOGRAPHY

I. DESIGN AND CONSTRUCTION

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SUMMARY

A new and experimental photodensitometer designed for quantitative chromatography is described. The principal features of the instrument were based upon the results of an extensive theoretical analysis and incorporate a mechanical arrangement for the production of a flying spot and an optical path in which two beams of light are separated after interaction with the medium. The device is constructed so as to be suitable for operation in the three principal modes; in reflectance measurements only the ratio of the beam signals is formed, whilst in transmittance measurements the ratio is converted to logarithmic form, in the fluorescence mode only a single beam is used.

The spectral range of the instrument extends from the red end of the visible spectrum to the medium ultraviolet, and quartz optics are utilized in most of the optic elements. A quartz halogen lamp and a xenon–mercury lamp may be used alternatively as the light source. Changeable interference filters are employed to determine the spectral position of the light beams and semiconductor photo-diodes with sensitivities extending into the ultraviolet are used as photo-detectors. In the determination of the sensitivity limits of the device the photo-diodes were replaced by photomultipliers and the apparatus was shown to fulfil most of the calculated theoretical predictions.

INTRODUCTION

This paper describes the principal features of the design of an experimental photometer for application in quantitative thin-media chromatography. The instrument was developed in our laboratories as a potential research tool for investigations into the biochemistry of the central nervous system. Extreme sensitivity coupled with high accuracy and good repeatability were the most important design targets. It was also thought desirable that the output of the instrument be available in a form which would be convenient both for immediate interpretation and for further analysis. Computer compatibility of the output of the instrument was the obvious answer.

A new instrument ought to be suitable for all the types of photometric determinations which are commonly employed in thin-media chromatography and related and similar techniques. In practice thi. means that measurements should be possible in both the transmission and the reflectance mode over a range of wavelengths extending from the red end of the visible spectrum to the near ultraviolet (UV). Conventional fluorescence and fluorescence quenching capabilities were also desired, though little emphasis has been placed upon the latter. Switching from one mode of operation to another should be easy and require as little manipulation as possible. When development of the device started, the principal chromatographic medium used by the presumptive user, was paper. The transport mechanism was designed therefore for this particular medium, clearly it is a relatively simple matter to modify the mechanism in order to accommodate all types of media.

INSTRUMENT

Principal features

The actual development of the instrument (see Figs. 1 and 8 for schematic representation) was preceded by an extensive theoretical study of the factors affecting the performance of photometric methods as applied to quantitative thin-media chromatography. The results obtained are largely published in a series of background papers by the authors[1–15]. A comprehensive survey showing the application of the theoretical conclusions to the conceptual design of the device will be published shortly. In many cases, it was found that the necessary techniques were available and well known in other fields of technology, but that they had to be adapted to the specific requirements of chromatography.

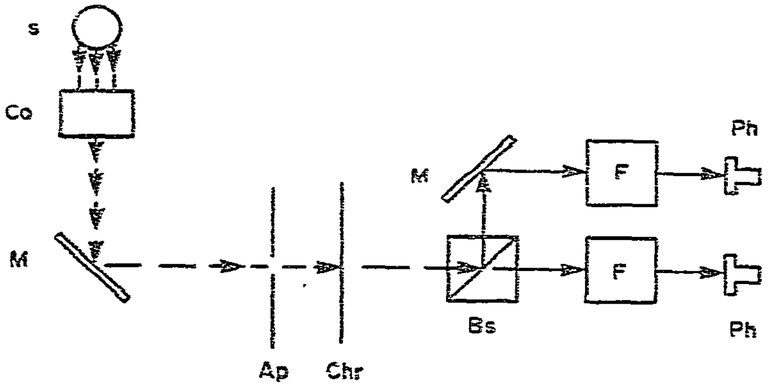

Fig. 1. Schematic representation of the optical path of a double-beam scanning device. s, Light source; Co, collimator; M, mirror; Ap, aperture; Chr, chromatogram; Bs, beam splitter; F, optical filter or monochromator; Ph, photo-detector.

Based upon these studies it was decided that for best performance the instrument should incorporate the following principal features: it should be a true double-beam instrument with only spectral (wavelength) separation between the two beams; in order to achieve this a unique arrangement of the optical system was developed, which permitted the avoidance of any spatial or time separation of the beams before they had interacted with the chromatogram. Equally important was the adoption of the principle of flying spot scanning. The resulting more complex mechanical and optical design was considered to be more than justified since there would be no need to worry about the concentration distribution within a measured zone. In order to achieve this principal, the two beam signals have to be linearized in terms of concentration before being combined and further processed. Of the simpler procedures available for this purpose logarithm forming for transmittance and simple inversion for reflectance measurements appeared to produce the optimum results[15]. For fluorescence analysis, linearization is not required[10,14].

The spectral range from the red end of the visible spectrum to the medium UV was covered by incorporating two light sources; a quartz halogen lamp for routine work in the visible part of the spectrum and a high-pressure mercury–xenon lamp for the UV. In place of the latter, other gas-discharge lamps, *e.g.* xenon, deuterium, could be used. The extension of the measuring range into the ultraviolet required the use of UV transparent material in most of the optical elements. As quartz was chosen for this purpose and since quartz optics are expensive, it became important to make the optical system as simple as possible. Because of this a relatively low efficiency of utilization of the available light ensued.

Semiconductor photo-diodes with extended UV sensitivity were adopted as photo-detectors. They were diodes of the Schottky Barrier (PIN) type operated in the photo conductive mode. Their spectral response curve is shown in Fig. 2b. With the use of these detectors the performance of the instrument became limited by electrical noise. This was due to the unexpectedly high efficiency of suppression of optical noise together with poor utilization of the available light. Photo-multipliers were then temporarily substituted to explore the performance limits of the method.

The formation of a ratio between the two beam signals after linearization was adopted is the most efficient method of reducing optical noise. The electronic circuits involved in the amplification and the processing of the photo-detector output signals are of the conventional type. Analog integration is carried out along the scanning lines (see Fig. 5) and the integrated output is recorded as a staircase curve on a strip chart recorder. The staircase representation (see Fig. 3) was chosen to make subsequent manual integration over the width of a zone easier. A smoothing filter was also provided; when optionally switched in, it produced the customary smooth shape (see Fig. 3b) of the output recording.

From the beginning, the need for further processing of the raw output data was recognized and it was decided that this should be achieved by computational procedures. An analog to digital (A/D) converter was, therefore, incorporated into the system. The A/D converter was interfaced with an IBM compatible digital magnetic tape unit so that a large computer (IBM 360) could be used for processing: this aspect of the analysis will be described in a subsequent publication. Experiments were also made with an all-digital electronics. The results were encouraging and will be used as a basis for future development.

Mechanical construction

There are many techniques known (and used) to produce a flying spot scan. The arrangement finally adopted is shown in Fig. 4. It consists of a metal drum with a helical slit, which revolves beneath a stationary rectangular aperture in the support

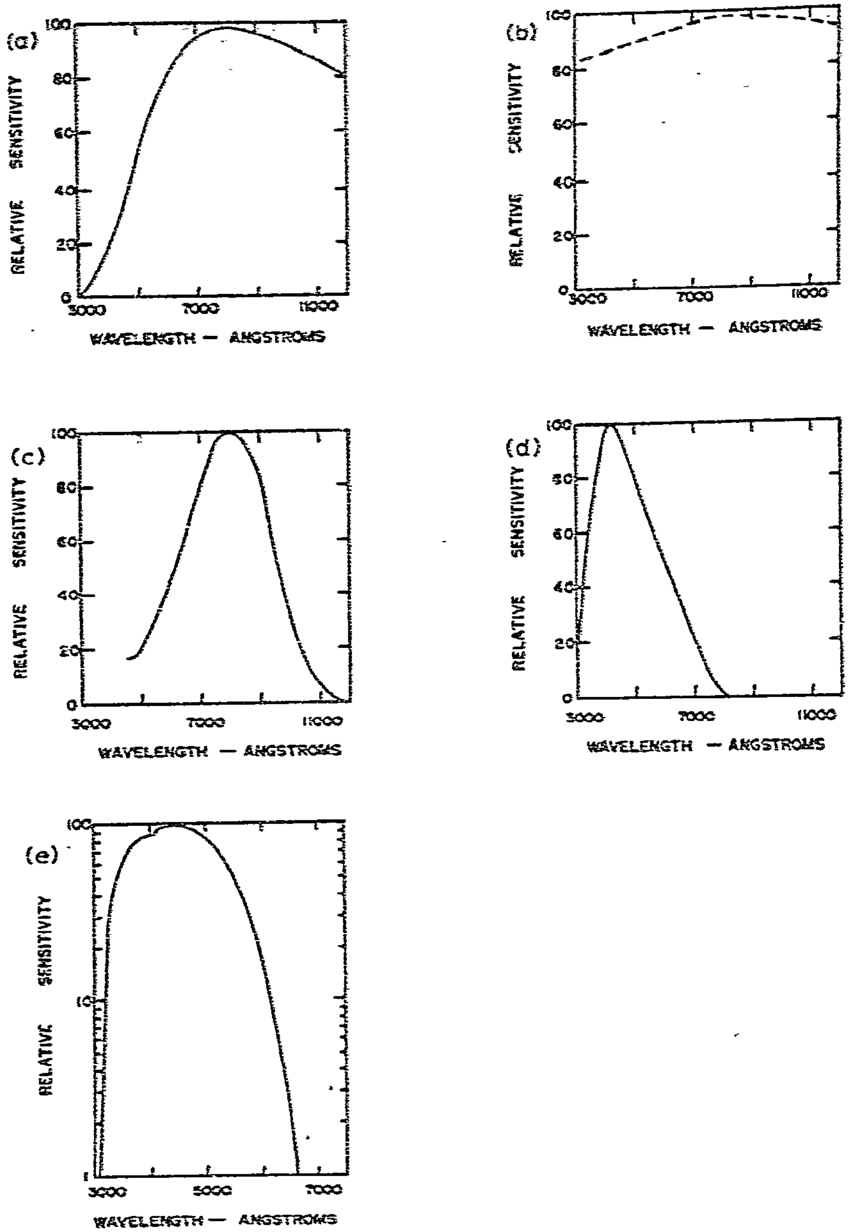

Fig. 2. Typical spectral response characteristics of different types of photo-detectors. a, Regular PIN diode; b, PIN diode with extended UV response; c, photo-multiplier with S1 photocathode; d, photo-multiplier with S20 photocathode; e, photo-multiplier with S11 photocathode.

table for the medium. A collimator and a cylindrical optical system images the light source upon the stationary aperture. The light can only pass through and reach the medium where the helical slit in the drum intersects with the stationary aperture. As the drum revolves, this spot moves with uniform velocity across the width of the medium.

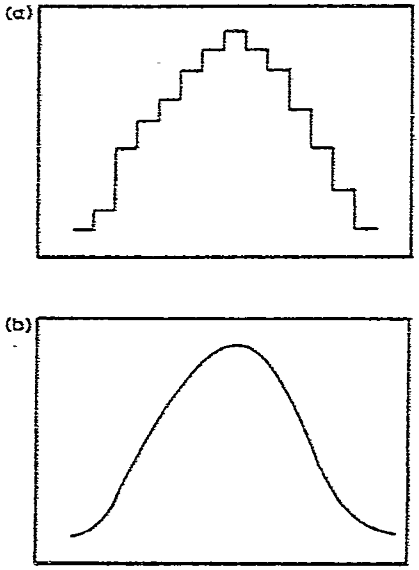

Fig. 3. Typical shape of an analogue output signal after line integration. a, Staircase; b, smoothed.

The chromatogram is clamped into a rectangular support frame, which can accommodate thin media up to 50 mm wide and 500 mm long. The medium is gripped only at the edges thus leaving nearly all its width unobstructed for the scanning beam. The support frame is moved longitudinally by a rack and pinion drive. Both the scanning drum and the pinion are driven via suitable gears from the same motor.

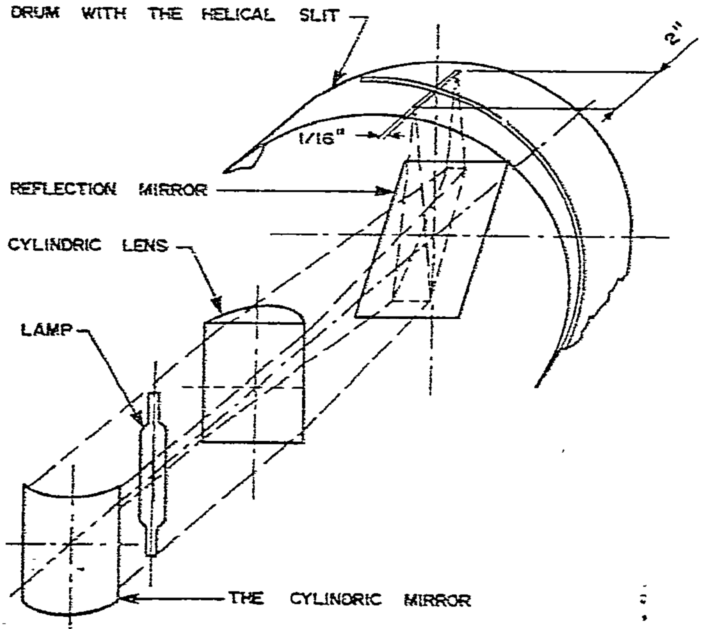

Fig. 4. Schematic diagram of a drum-type flying spot arrangement.

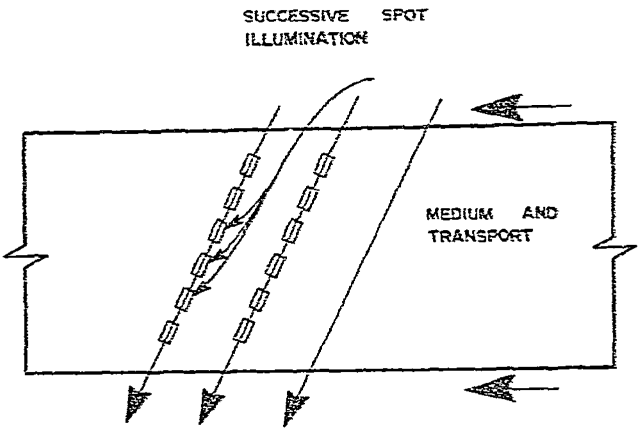

Fig. 5. The trajectory (exaggerated) of the scanning beam on a chromatogram.

The gear ratios are chosen so that the whole exposed area of the medium is covered by the scan (see Fig. 5). If desired, the transport of the medium can be disengaged, leaving the scan line in a stationary position.

Illuminating system

The two lamps used possess different electrical characteristics and are supplied, therefore, by independent power supplies. Both supplies are carefully stabilized, although because of ratio forming, the system is much less sensitive to variations of the light output of the lamps than is the case in all previously described scanning devices. The two lamps along with their respective collimating mirrors and lenses are housed in a T-shaped attachment to the main body of the instrument. A two-position mirror is used to switch between the lamps (see Fig. 6). A heat filter is provided to protect the rest of the optical system from the heat generated by the lamps. A cylindrical quartz lens produces a rectangular shaped image of the light source in the plane of the support table of the medium. The size of this image is approximately equal to the size of the slit-shaped aperture in this table.

Provision has also been made to insert wavelength-selective or polarizing filters into the path of the illuminating light. Wavelength selection on the primary side is desirable for fluorescence measurements; polarizing filters may be of advantage for reflectance measurements.

Secondary optics

On the secondary side, that is after interaction of the illuminated beam with the chromatogram, different optical systems are used for measurement from the illuminated and from the non-illuminated sides of the medium. The latter will be described first.

Most chromatographic media have relatively little absorption but exhibit strong scattering. The light flux transmitted or reflected from such a medium has an intensity distribution, which follows essentially a cosine (Lambert's) law (Fig. 7). The flux density decreases approximately with the square of the distance from the radiating

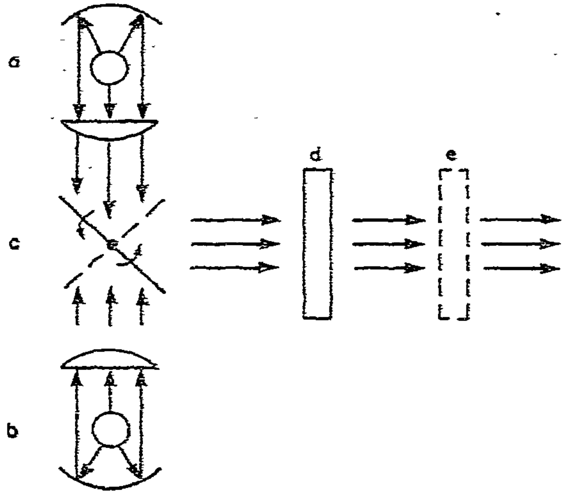

Fig. 6. Schematic representation of the lamp housing. a, Quartz halogen lamp; b, xenon–mercury high-pressure lamp; c, two-position mirror for lamp switching; d, heat filter; e, optional additional filter.

surface element. These factors have to be kept in mind when determining the optical density of such a medium.

For efficient utilization of the available light a collecting lens possessing a large diameter and placed close to the surface of the medium is required. Its purpose is to produce a much reduced size image of any point on the scanning line upon the sensitive surface area of the photo-detector. The noise contribution of most photo-detectors is proportional to the square root of their light-sensitive area. A substantial reduction in the width of the image mainly in the transversal direction is, therefore, desirable. A combination of spherical and cylindrical lenses is required to achieve

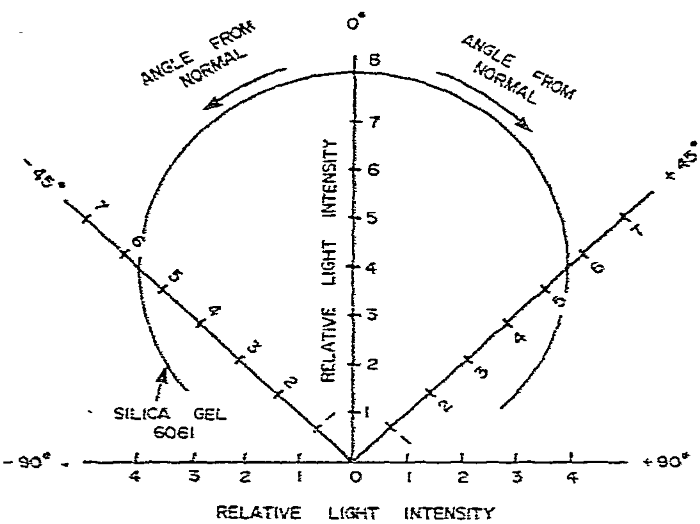

Fig. 7. The cosine (Lambert's) distribution.

this goal. In order to possess a reasonably uniform light-collecting efficiency regardless of the position of the scanning spot across the medium the main collecting front lens was ascribed a diameter some 30% larger than the maximum width of the medium. It was made circular in shape, though a rectangular shape would have been equally satisfactory. The uniformity of light collection achieved across the width of the slit was quite satisfactory, but the total efficiency fell short of expectations. A Fresnel lens system could be expected to provide a superior performance.

From the collecting lens system the light is directed towards a beam splitter–mirror system, which divides the light beam into two separate beams with approximately equal intensities. One of the two beams separated in this way becomes the principal measuring beam whilst the other one becomes the reference beam. Interference filters can be inserted into the paths of these two beams in order to select their required spectral position. Space is also provided for the addition of other filters such as a neutral density or polarizing filter. An additional small lens then focuses the image of the scanned slit upon the two photo-detectors (Fig. 8).

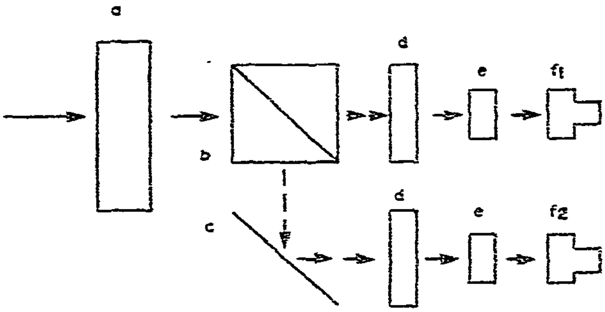

Fig. 8. The optical beam dividing arrangement (schematic) on the secondary side. a. Collecting lens; b, beam splitter; c, fixed mirror; d, filters; e, image focussing lens; f_1 and f_2, photo-detectors.

Although the double beam method is also advantageous for fluorescence measurements, the combination of the two beam signals in such a way as to combat efficiently optical noise, is much less straightforward. As the theoretical analysis for this particular case had not reached a sufficiently advanced stage to be included in the instrument design, fluorescent zones are measured using a single beam in the conventional manner.

For measurements from the illuminated side, whether they be of the reflectance or fluorescence type, the photo-detector filter arrangement is maintained, but means have to be provided to collect the light returned from the illuminated surface of the medium and to guide it to the photo-detector filter assembly. After some experimentation with a beam splitter–mirror system, the alternative of a fiber optic light guide system was adopted instead. Though mechanical considerations prevented the use of a large bundle cross-section, its entrance aperture could be placed very near to the illuminated surface thus compensating for any loss of efficiency due to the small collecting aperture available and to the rather crude lens systems placed at the entrance and exit of the bundle. Since imaging is not required a non-coherent bundle was used. The bundle was first randomized and then divided into two halves, thus replacing the beam splitter mentioned above. The light leaving the exit apertures of the bundle were crudely collimated and then passed through the same filters as used in the transmittance arrangement and finally focused upon the photo-detectors.

The wide range of light intensities that can be accommodated by semiconductor photo-detectors without overload or impairment of their linearity of response obviates the need for an optical sensitivity control. Such an advantage does not obtain in the case of photo-multipliers where neutral density filters are required in order to adjust the intensity of the light reaching the photo-multipliers to an optimum level. An electrical overload protection is however provided, so that damage to the photo-detectors does not occur following excessive illumination. It should be pointed out that the photodensitometer described in this paper was designed to use semiconductor photo-detectors; photo-multipliers were only used when it was discovered that the performance of the device was limited by the electrical noise of the photo-detectors, not the optical noise as had been originally anticipated. This was due partly to the unexpectedly efficient cancellation of the optical noise and partly to the relatively poor performance of the optical system. Photo-multipliers were provided and used in order to test the full capabilities of the system. The performance data reported in the following paper refer, therefore, to the photo-multiplier version.

Chopping frequency

In instruments of the kind described above it is customary to interrupt (to chop) the light beam at regular intervals. Chopping is usually achieved by mechanical means, in this case by a chopping drum with regularly spaced slits, which revolves concentrically and synchronously with the scanning drum (see Fig. 4). The chopping frequency is determined by the revolving speed of the drum and the number of slits it carries. This frequency is not chosen arbitrarily but is determined largely by the noise characteristics of the photo-detector. Chopping the light beam at regular intervals amounts to what, in telecommunications technology, is called modulation. The signal produced by the light beam is originally d.c. in character. By chopping it is shifted to a higher frequency position symmetrically centered around the chopping frequency. This is shown schematically in Fig. 9. It is a well known fact that for optimal noise performance, photo-detectors of any kind should operate above a certain minimal frequency, which is called the cross-over frequency of the device. The cross-over frequency is dependent upon the manufacturing technology and may, therefore, vary from type to type; it must be calculated from the manufacturer's specifications. The width of the side bands in Fig. 9 is largely dependent upon the scanning speed and the size of the flying spot. In this case, a relatively coarse raster possessing 35 spots per scan line was adopted. With a scanning speed of four lines per second this resulted in 140 spots per second corresponding to a minimum practical width of each side band of about 100 Hz (70 Hz theoretically).

Based upon these considerations a chopping frequency of 450 Hz was chosen as a compromise between mechanical and electrical requirements. Experience later showed that this choice was on the low side and values of the order of 800 Hz would have been preferable.

Electronics

The output signal from the photo-detectors is fairly small and must therefore be suitably amplified in order to permit further processing. Signal level controls are

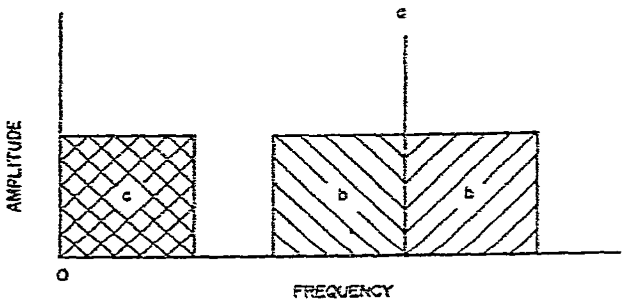

Fig. 9. Frequency spectrum of the photo-detector signal with beam chopping. a, Carrier (chopping) frequency; b, side bands; c, spectrum after demodulation and smoothing.

also provided at this point. Since the light beam is chopped, the signal is essentially a.c. and many stability problems connected with the amplification of low-level d.c. signals are thus avoided. The original signal, however, must be restored and this is done by a subsequent full-wave rectifier bridge (see Fig. 10). A band pass filter ahead of the rectifier reduces the noise content, whilst a low pass after rectification smoothes the signal by removing the remainders of the chopping frequency. The width of the pass band of these filters is determined by the previously mentioned width of the side bands of the modulated signal.

The further paths of the signal are different depending upon the different principal modes of operation. For fluorescence measurements irregardless of the side of the medium from which they are taken, only the signal of the principal measuring beam is employed. It is passed via a buffer amplifier to an analog integrator. The integrator is essentially a high-gain d.c. amplifier with a resistive–capacitive feedback

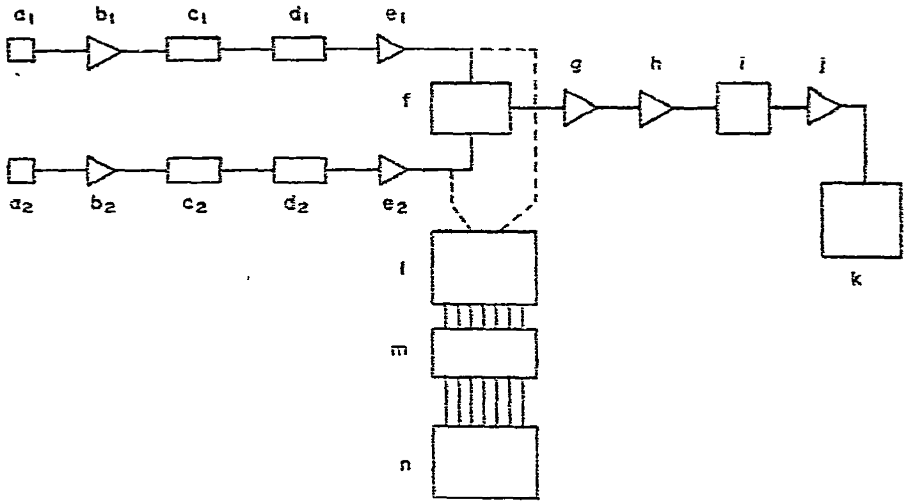

Fig. 10. Block diagram of electronics. a_1 and a_2, Photo-detectors; b_1 and b_2, band pass filters; c_1 and c_2, full wave rectifier bridges; d_1 and d_2, low pass (smoothing) filters; e_1 and e_2, buffer amplifiers; f, analogue divider; g, logarithmic amplifier; h, integrator; i, sample and hold; j, buffer amplifier; k, analogue recorder; l, analogue → digital converter; m, magnetic tape unit interface and control; n, magnetic tape unit, computer compatible.

network between output and input. The integrator is started at the beginning of each scan line by a trigger pulse derived from the position of the scanning drum. It integrates the signal over the duration of one scan line, at the end of which it is again reset. The value reached is held in a sample and hold circuit until the end of the following integration period. From the sample and hold circuit it is passed on via a buffer amplifier to the output. In this way a staircase output wave of the shape shown in Fig. 3 is produced. The height of each stair corresponds to the integral of the fluorescence signal over the last scan line. A standard analog recorder is used to display the output. If a smooth output curve is desired, a smoothing filter can be inserted into the output circuit ahead of the recorder.

For conventional transmittance and reflectance measurements both beam signals are utilized. After rectification, filtering and amplification they are fed to an analog dividing circuit, which produces the ratio $S(t)_R/S(t)_M$ where $S(t)_R$ and $S(t)_M$ are the instantaneous values of the beam signals from the reference beam and the measuring beam respectively. In the reflectance mode of operation the resulting ratio is passed on to the integrating stage and recorded in the manner described above. In the transmittance mode, a logarithmic amplifier is interposed between the ratio forming circuit and the integrator.

The purpose of logarithm and/or ratio forming is twofold. Firstly, the optical noise is considerably decreased and the signal made nearly independent of fluctuations of the output of the light source. Secondly, a signal is obtained which is a closely linear function of the concentration. It is only on this basis, that a flying spot system can operate efficiently. It should be noted that linearization must be performed before integration. At extremely low concentrations the output signal is always a closely linear function of the concentration[1] in which case linearization of the output is not required. Compensation of the optical noise then becomes the most important purpose of the described circuits.

Digital processing

It has already been mentioned that digital processing of the raw data supplied by the photometer improves, in many respects, performance and efficiency of the scanning technique. To render this possible without the need for external peripheral equipment an analog to digital (A/D) converter has been built into the device. This device converts the output signal into a string of 10-bit digital words, which are then stored on a computer compatible magnetic tape. The written tape unit serves as input medium for further processing on a suitable computer.

The digital part of the photometer is interfaced fully with the magnetic tape unit used. In view of the low speed of data generation, paper tape storage would be just as feasible. Interfacing with a low-cost mini-computer for on-line processing was contemplated, but not implemented at this time.

Construction

The experimental instrument was built as two units: one unit contains the mechanical and optical parts and is housed in a light-tight casing in order to provide access to the transport mechanisms to facilitate easy loading and unloading of the chromatograms.

The electronics section was built as a separate unit; it is connected by cables to the optical part of the system so that all the electrical controls are located on the front panel of the first unit. The electronic circuitry was built throughout from integrated circuits interconnected by a wire-wrapping technique. The preamplifiers for the photo-detectors were not included in the electronic unit; instead these were placed in shielded enclosures as close as possible to the photo-detectors. The power supply units for the illuminating lamps were also mounted in a separate location.

CONCLUSIONS

The scanning system described in this paper was designed and built as an experimental unit in order to verify general concepts as reported in previous publications[1–15] and to test several techniques that are new in this field. The system was subjected therefore to many changes, as new information and experience was gained. In its penultimate version, the device was tested extensively. The results of some of these trials are reported in the following paper[16]. At this stage it is appropriate to state that although not all of the original expectations were met, the instrument performed well and is probably superior to any other comparable device. The predictions of the theory, on which the design was based, were verified in all cases; implementation however sometimes fell short of the theoretical ideal. A new device, more suited to commercial reproduction, is now under development. The design of this device is based upon the experiences obtained with the above described instrument. It is hoped that the various shortcomings discovered in working with the experimental model will be avoided or circumvented.

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