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BACKGROUND THEORY FOR THE DESIGN OF A PHOTOMETRIC INSTRUMENT FOR QUANTITATIVE THIN-MEDIA CHROMATOGRAPHY

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SUMMARY

This paper surveys in a comprehensive fashion those theoretical considerations upon which the design of a new experimental photometer for quantitative chromatography has been based. High sensitivity and good accuracy were the principal aims. Transmittance, reflectance and fluorescence measurements were considered. The principle of flying spot scanning was adopted because it makes the method independent of zone geometry. For subsequent integration a linear relationship between photometer output and concentration is necessary. Logarithm forming for transmittance and simple inversion for reflectance were found to provide the required linearity over a wide range of concentrations and for most chromatographic media. Fluorescence does not require linearization. The ultimate limitation in performance is determined by noise. If sufficient light is available, electrical noise can usually be disregarded; optical noise then becomes the decisive factor. Following a brief discussion of the various sources of optical noise the double-beam principle was introduced as the most efficient means to combat optical noise. Ratio forming of the two beam signals is in general superior to difference methods. In the ideal case the double-beam system should provide a smooth base line affected only by electrical noise. In practice, however, some residual optical noise always remains. The signal-to-optical noise ratio obtained during measurement of absorbing zones is constant and independent of the signal amplitude. Methods of processing the signal once it has been obtained and the potential for further noise reduction are briefly discussed. This subsequent processing by digital techniques is considered to be the most advantageous procedure. Finally some of the possibilities offered in the area of photometric scanning by tunable lasers are briefly mentioned.

INTRODUCTION

This paper presents a summary of the theoretical background considerations which were at the root of the development of an experimental high-performance photometric device for the quantitative assessment of thin-media chromatograms. The

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instrument itself is described in a separate publication[1]. That paper provides a condensed but quite comprehensive survey of the problems which have to be solved in order to meet high-performance criteria in this area. It is also a demonstration of the potential of interdisciplinary teamwork, showing how established knowledge from one field of science (in this case from communication theory) can be successfully applied to the solution of problems in a seemingly completely different discipline.

The instrument to which the arguments presented specifically apply was conceived as a research tool for investigations of the biochemistry of the central nervous system. Extreme sensitivity, good reproducibility and high accuracy were the performance criteria adopted. In addition, the instrument should be suitable for all common kinds of photometric measurements used in thin-media chromatography and related fields. More specifically it should permit measurements in both the transmission and the remission modes using wavelengths extending from the medium UV to the near IR. Fluorescence measurements should also be possible from either side of the medium using selectable exciting wavelengths. Fluorescence quenching was also included, but considered as a method of secondary importance for the applications envisaged.

FLYING SPOT SYSTEM

Almost any kind of photometric method relies at some stage on the conversion of a measured light intensity to an electric signal. The conversion is produced by an element called a photodetector. A vast range of photodetectors using a wide variety of physical principles are available today. In most cases they produce a response which is linearly dependent upon the total light flux incident upon the photodetector. But not all this flux provides useful information. In chromatographic applications only those changes in light flux due to the presence of a separated substance on the chromatogram are useful.

The useful part of the output signal of the photodetector is in general a linear function of the change in the total amount of light arriving at the photodetector. If the intensity of this light flux is spatially non-uniform, the photodetector acts as an integrator, which averages the signal intensity $I(f)$ over the whole area F that it "sees".

$$E(F) = K \int_F I(f)\mathrm{d}f \tag{1}$$

Here $E(F)$ is the photodetector output signal and K a proportionality constant. In general, the light intensity $I(f)$ varies in a nonlinear fashion with the spatial concentration $c(f)$ of the measured substance on the chromatogram[2,3].

$$I(f) = \varrho[c(f)] \tag{2}$$

It is the concentration $c(f)$ that the chromatographer is interested in. Introducing eqn. 2 into eqn. 1, it becomes clear that the photodetector signal is a unique function of $c(f)$ only if $c(f)$ is constant over the whole area F.

$$c(f) = a = \text{const.}$$
$$E(F) = K \int_F \varrho(a)\cdot\mathrm{d}f = K\cdot F\cdot a \tag{3}$$

As a consequence of diffusion processes occurring during development the spatial rate of change of concentration is usually quite small. The validity of eqn. 3 can then be enforced simply by making the area F "seen" by the photodetector at any given time small with respect to the diffusion limited size of the zone of the separated substance on the chromatogram. For complete coverage of the whole area of the chromatogram the illuminated area must move in two dimensions with respect to the medium. In technical terms a system of this kind is called a "flying spot" system.

If the area seen by the photodetector is larger than the diffusion limited area of constant concentration, for example, when conventional slit scanning is employed, special steps must be taken to insure that condition 3 is maintained. No such special precautions are required for a flying spot arrangement.

The arguments as outlined above in favor of flying spot scanning do not apply to fluorescence measurements. The light intensity received by the photodetector in this latter case is a linear function of concentration[4,5] and eqn. 3 is satisfied regardless of the size and the shape of the scanning aperture. If the width of the zone along a scan line is small compared to the total length of the line, flying spot scanning provides improved contrast and amplitude resolution. This advantage applies to all modes including fluorescence.

LINEARIZATION

What the chromatographer usually requires to know is the total amount of investigated substance Q in a given zone of area Z. Z is almost always much larger than the elementary scan area F. To obtain Q summation (integration) of $c(F)$ over the whole area of the zone is required.

In a flying spot system (and in appropriate situations also in a slit scanning system) the output signal $E(F)$ of the photodetector is a unique, but in general nonlinear function of $c(F)$. To make integration without excessive error feasible, $E(F)$ must be converted to a form which is a closely linear function of $c(F)$.

The deviations of $E(F)$ from linearity with respect to $c(F)$ are different depending upon the kind of photometric procedure used. In the case of fluorescence measurements the linearity is in most cases excellent[4,5] and no conversion operations are required, regardless from which side of the medium the fluorescence is measured.

Fluorescence quenching requires linearisation similar to transmittance, but it has not been analysed in detail.

For transmittance measurements logarithmic conversion of the photometric output signal can be shown to provide (with most presently employed chromatographic media) a signal which is a closely linear function of concentration over a fairly wide range of concentrations[6].

$$\log E(F) \approx k_1 \cdot c(F) \tag{4}$$

For reflectance measurements logarithm forming is not a satisfactory method of linearisation. Simple inversion of the photodetector output signal gives much superior results[6,7].

$$\frac{1}{E(F)} \approx k_2 \cdot c(F) \tag{5}$$

In both cases linearisation has to be carried out before integration. The operation defined by eqns. 4 and 5 can easily be implemented both digitally or by analog circuits.

For more demanding applications, where the degree of linearity provided by the two elementary operations quoted above is not satisfactory, a more sophisticated, but still relatively simple approach may be used. The non-linear response

$$E(F) = \psi[c(F)] \tag{6}$$

can be expanded into a power series around the point $c(F) = 0$. The series can be truncated after a few terms and the resulting polynomial in $c(F)$ inverted by a computer. Alternatively an electrical model concept[2,3] can be used to design an analog system of linearization. In the light of our experiences to date, however, we have concluded that this sophistication is hardly ever warranted, at least not for applications in chromatography. No linearization regardless of the measuring mode employed is required for extremely low concentrations.

ELECTRICAL NOISE

Besides their useful and desirable response to incident light all known kinds of photodetectors generate also an undesirable random noise signal, which is largely independent of the intensity of the incident light and mainly a function of the design of the photodetector element. Though noise is generated in every element of the electronic circuitry associated with the amplification and the processing of the photodetector signal, it is almost always the photodetector noise which determines the overall level of electrical noise in the system.

Photodetection noise is commonly expressed in terms of the power of a light flux impinging upon the photodetector, which produces an electrical output with the same r.m.s. value as the noise signal[8]. This power is called the noise equivalent power (NEP) of the photodetector. The NEP value of a particular type of photodetector increases in general approximately with the square root of the sensitive area of the photodetector.

It is, therefore, desirable to use the best possible collimation of the incident light beam and the smallest photodetector size compatible with the beam cross-section.

For reasonable results the useful component of the light signal impinging upon the photodetector (that is the decrement in light flux due to the presence of measured substance) must be several times larger than the NEP value. It is, therefore, the noise equivalent power and not the conversion sensitivity (responsitivity) of the photoelement, which limits the sensitivity of the method.

The amplitude of the noise contributed by the photodetector increases with the square root of the electrical signal bandwidth. Electrical filters are, therefore, usually provided, which reduce the bandwidth of the processed signal to the minimum compatible with the required speed of response. The higher this speed, the more bandwidth is required. The NEP value in manufacturer's specifications refers usually to a signal bandwidth of 1 Hz and to the peak of spectral response. At other wavelengths of the illuminating light the NEP figure may be considerably worse.

An example will help to illustrate these considerations. Let us assume that the specifications of the instrument require a sensitivity threshold of 0.002 optical density units. This represents a measurable change of approximately 0.5% of the light

intensity reaching the photodetector. The corresponding spatial concentration of measured substance depends, of course, upon its absorbance. The NEP of the chosen photodetector as specified by the manufacturer is $5 \cdot 10^{-14}$ W·Hz$^{1/2}$. This is a typical value for contemporary high-grade semiconductor devices; at the blue end of the spectrum the responsitivity may be decreased by a factor of about 3. The required bandwidth is 100 Hz. The corresponding equivalent light input is $\approx 1.5 \cdot 10^{-12}$ W. For good performance the electrical signal-to-noise ratio should be of the order of 10 or better. The required change in light intensity is then $1.5 \cdot 10^{-11}$ W and the total light intensity reaching the photodetector should be $>7.5 \cdot 10^{-8}$ W. Small as this value may appear, it is not easy to obtain in practice. Nearly all photodevices exhibit a considerable increase in intrinsic noise at very low signal frequencies. To avoid this so-called flicker phenomenon it is customary to interrupt (to chop) the light beam at regular intervals with a frequency of several hundred Hz. The chopping frequency acts as a carrier for the useful signal, shifting its frequency position away from the flicker noise region. An additional advantage of this technique is that the first amplifier stages following the photodetector can be capacitively (a.c.) coupled. However, before the final processing and display can take place, rectification and smoothing is required in order to restore the original shape of the signal.

Photomultipliers versus solid state devices

For applications in chromatography two fundamentally different kinds of photodetectors are available. Photomultipliers, the traditional choice, have the advantage of a large built-in gain requiring little subsequent amplification. Their noise equivalent power is very low; values of 10^{-20} W·Hz$^{1/2}$ are obtainable with high-performance models. The spectral response characteristics are in general, however, less than ideal for applications where a broad range of wavelengths must be encompassed. A further drawback is their sensitivity to accidental over-exposure. Other weaknesses are limited life time, aging, a relatively costly power supply and special requirements with regard to mounting.

Semiconductor photodetectors in recent years have been able to challenge the photomultipliers and they are slowly beginning to encroach upon those areas traditionally reserved for photomultiplier tubes. At the present stage of development, their NEP figures are about at par with low- to medium-grade photomultiplier tubes. With the exception of the recently developed avalanche type devices, they do not possess built-in gain and their output signal is, therefore, small, requiring extensive amplification. On the other hand, however, they are much more rugged and undemanding with regard to mounting techniques and associated power supply. They exhibit excellent linearity of response over many decades of illuminating light intensity and over-exposure does not in general produce permanent damage. They are available with reasonably flat spectral response characteristics covering in one element the whole range from the near IR through the visible part of the spectrum and into the medium UV.

Summarizing, it would appear that solid state photodetectors are preferable to photomultipliers. They require, however, careful design of the optical part of the instrument in order to maintain the necessary high level of illumination.

OPTICAL NOISE

The effect of electrical noise can be made almost arbitrarily small by making the illuminating light intensity large and/or by choosing detector elements with very low NEP figures. It is then the optical noise which limits the results that can be obtained. Keeping the optical noise low is of paramount importance therefore in the design of a high-performance instrument[9,10].

There are several sources of optical noise; their relative importance varies depending upon the selected mode of operation. In chromatographic applications it is only that light which has penetrated into the interior of the medium that carries useful information. Light which has undergone specular reflection at the surface does not provide any such information and is, therefore, useless. The surface reflectivity of most chromatographic media is, however, a random variable in space with a mean value $\bar{\varepsilon}$ (ref. 7). As a result, the intensity of the light entering the medium exhibits random fluctuations from point to point. These fluctuations appear as optical noise and they affect almost to an equal degree the three principal modes of operation, that is, transmission, remission, and fluorescence.

When the remission mode is employed, part of the specularly reflected light may reach the receiving photodetector where it produces an additional noise signal. It can be reduced by careful optical design, but cannot be totally suppressed.

Another source of optical noise and frequently the most serious one is caused by local variations in the thickness of the chromatographic medium. It can be shown that these fluctuations mainly affect measurements in the transmission and in the fluorescence modes[6,7]. The remission mode is less affected the larger the optical density of the medium.

A very similar effect is produced by spatially random fluctuations in the optical parameters of the medium. These may be caused by density fluctuations, non-uniform particle size, localized changes in composition etc. and in contrast to thickness fluctuations they affect all modes of operation to an approximately equal degree[7].

The treatment of the medium during the chromatographic process sometimes produces systematic, non-random changes in its optical parameters. These are usually only of significance at extreme sensitivity levels; their main effect is a distortion of the scanning baseline, which assumes a slanting or curved appearance.

Specific for fluorescence quenching is noise due to fluctuations in the response of the fluorescent component of the medium. This phenomenon is similar to noise produced by non-uniformity of the medium itself.

Optical noise can also originate in the light source. Stabilization of the supply voltage is helpful principally in the case of incandescent lamps. With gas discharge lamps stability of the discharge is an important factor. This depends mainly upon the design of the lamp and care is therefore required to select a suitable low-noise type. Though not really a source of noise, aging of the light source must also be considered. Most affected in this regard are the high-temperature incandescent lamps. Changes in the color temperature of these lamps may bring about the need for frequent recalibration both in single-beam and in double-beam instruments.

Optical noise is the most serious single factor which has to be dealt with in order to improve the performance of photometric methods for quantitative thin-media chromatography. The most efficient approach in overcoming this problem is

the double-beam principle. Though many commercial instruments are today referred to as double-beam devices, only a few of them can be considered to be true double-beam systems.

As indicated by the name, a double-beam instrument employs two separate beams of light usually derived from the same source. The beams can be separated spectrally, in space or in time. For the purposes considered here wavelength separation is the method of choice. It can be shown that separation in space and time should be kept to a minimum or be avoided altogether.

With wavelength separation the energy maximum of the principal measuring beam is chosen to coincide with the peak of the absorption spectrum of the substance under investigation; the second reference beam is centered at a wavelength where examined substance has little or no absorbance. Strict mono-chromaticity is not required, though the spectral width of both beams should be narrow compared to the width of the absorption band of the substance analyzed[11]. It has to be kept in mind that monochromaticity is in general attainable only by a large loss in illuminating light energy. The use of a tunable laser would circumvent this difficulty; with the present state of laser technology, however, this is too expensive an alternative. To insure uniform interaction of the two beams with the blank medium, a feature which is essential for good noise suppression, the spectral separation of the beams should be not more than absolutely necessary. For the very same reason spatial separation of the beams is undesirable, since it is bound to lead to differences in the beam–medium interaction. For best compensation of the optical noise, the beams should at any time interact with the same volume element of the chromatogram. Such an arrangement is shown schematically in Fig. 1.

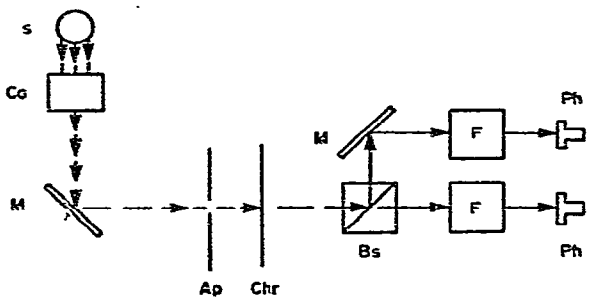

Fig. 1. Optical path of a double-beam system with spectral separation only (schematic). S = Light source; Co = collinator; Ap = aperture; Chr = chromatogram; M = mirror; F = optical filter/monochromator; Bs = beam splitter; Ph = photodetector.

Separation in time is frequently used in double-beam instruments and vibrating mirrors, chopper discs and similar devices have been employed for this purpose. These systems perform well if the medium is fairly stationary during the period of the beam-switching cycle. This can be achieved either by a step drive mechanism of the medium or by using a high frequency of beam switching. If this condition is not met, the performance of the system deteriorates in the same way as with spatial separation.

Combination of beam signals

To produce the desired effect the signals derived from the two beams must be

combined in a way which provides maximum suppression of the optical noise without affecting the useful information. Two elementary operations are available for this purpose, *viz.* subtraction and division.

Both operations are effective, but a detailed analysis[7] shows that division is the superior approach. It not only provides somewhat better noise suppression, but results in an output signal which is nearly independent of the intensity of illumination. Stabilisation of the light output of the source becomes, therefore, not critical as long as the relative intensity of the two beams remains constant.

For best performance of either method linearization of the beam signals is desirable before recombining them[7]. In the transmission mode logarithm forming is the most effective elementary method of linearization. However, the difference of two logarithms is equal to the logarithm of their ratio:

$$\log s_1 - \log s_2 = \log \frac{s_1}{s_2} \qquad (7)$$

Noise cancellation by subtracting the two logarithmised signals is only slightly less effective than forming their ratio $\log s_1/\log s_2$ and is in most other respects equivalent to ratio forming; it is superior with regard to elimination of fluctuations of the illuminating light source. It is, in addition, easier to implement and thus permits the use of almost identical flow diagrams for the processing of transmittance and reflectance signals (see Fig. 2). At extremely low concentrations where linearization is not required[6], the logarithmic converter can be eliminated even in the transmittance mode.

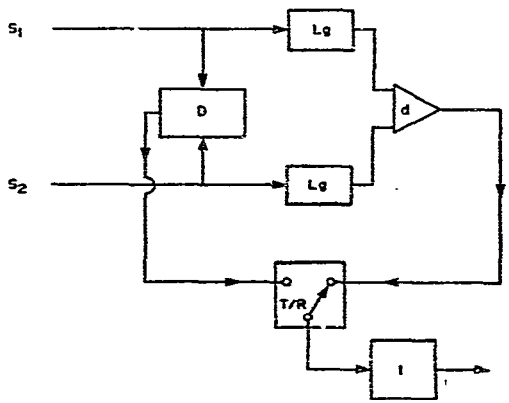

Fig. 2. Block diagram for the linearization and combination of the two beam signals in a double-beam system (schematic). At extremely low concentrations the reflectance position of the switch T/R is with advantage used also for transmittance. s_1, s_2 = Preprocessed beam signal input; D = dividing (ratio forming) circuit; Lg = logarithmic converter; d = difference amplifier; T/R = transmittance–reflectance switch; I = integrator.

Baseline noise

In the ideal case both difference and ratio forming of the two beam signals should provide a smooth baseline only very slightly crinkled by the electrical noise of the photodetectors. It should be noted, however, that the double-beam system does

not reduce electrical noise which affects both beam signals in an uncorrelated way; in fact, the electrical noise is increased by a factor of $\sqrt{2}$.

The term "baseline" refers here to those sections of the output signal which arise from parts of the medium where the concentration of investigated absorbing substance is zero. In reality, of course, ideal optical noise cancellation does not occur even for the baseline. There are several reasons for this. The two beams are not ideally symmetrical and may interact even with the blank medium in slightly different ways, and the circuits which process the two beam signals and recombine them similarly are not ideal. In this respect, digital methods are better than analog ones. Finally, in a theory[7] based upon a linear approximation, second and higher order optical effects which are present are disregarded.

Even an ideally smoothed baseline is no proof of noise-free measurement. In the case $c(F) \neq 0$ the noise cancellation even theoretically calculated is imperfect. This means that deviations from the baseline are affected by an error due to the residual optical noise. It can be shown that the ratio of the useful signal, represented here by the deviation from the baseline, to the residual noise is constant and independent of the concentration. The percentage ambiguity of a reading is, therefore, at least in theory, constant over the whole range of measurable concentrations. This is an important difference from most other measuring problems, where the error due to noise decreases when the value of the measured parameter increases.

Since the optical signal-to-noise ratio remains ideally constant, regardless of the concentration encountered by the beam, the sensitivity and resolution limits obtainable are determined by the residual noise of the signal. The factors responsible have been discussed above. Circuit errors, beam asymmetry and electrical noise can, by proper design, be reduced almost arbitrarily, though sometimes only at considerable expense. This leaves higher order optical effects as the main limitation to an increase in detection sensitivity, which may be difficult to overcome.

In the fluorescence mode the signal amplitude in the absence of investigated substance is very small. The baseline is, therefore, almost ideally smooth even if only a single-beam instrument is used. It is, therefore, frequently concluded that fluorescence measurements are not affected by optical noise. From the arguments above, however, it becomes evident that this assumption is erroneous. Deviations from the baseline are in the fluorescent mode affected in the same way by optical noise as with any other mode of operation. To reduce the resultant error, a double-beam system even in this case would also be highly desirable. However, implementation of a double-beam system for fluorescence measurements is much less straightforward than for direct measurements by transmission or remission. Some considerations pertinent to this problem will be reported in a separate paper.

The sensitivity of fluorescence measurements is largely limited by residual fluorescence of the medium and by the electrical noise of the photodetectors.

The total accuracy of photometric determinations is determined by the error in determining the exact position of the baseline and the error of the deviation from the baseline in the presence of separated material. The two errors can be considered uncorrelated and hence additive according to a square law. The mean compounded error thus becomes

$$\sqrt{(\varepsilon_1^2 + \varepsilon_2^2)} \qquad (8)$$

SUPPLEMENTARY PROCESSING

Most commercial instruments display acquired raw data in analog form on a strip chart recorder. Supplementary processing, however, can considerably reduce residual noise and thereby improve the performance parameters of the method[12].

To determine the quantities of separated substances on a chromatogram the area enclosed under the individual peaks of the recording trace must be found by integration. Frequently this is done by hand, but automatic integration by digital techniques has become both feasible and economic.

Integration can be shown to be equivalent to a kind of low pass filtering. The amplitudes of both electrical and optical noise increase with bandwidth, though in different ways. Integration, therefore, decreases the noise content of the signal without affecting its useful component. The smoothing effect of integration increases approximately with $\sqrt{(Z/F)}$, where Z is the area of the respective zone on the chromatogram and F the area of the scan. Obviously, a quite significant improvement in signal-to-noise ratio may be obtained in this way.

Integration can also be used to smooth the baseline. If performed manually, the procedure is extremely tedious and time consuming. Simply replacing the noisy baseline by a straight line drawn "by eye" possesses the danger of introducing arbitrary errors, which are difficult to assess. Digital filtering appears to offer the best solution. Its advantage is that rather sophisticated methods of smoothing can be used without appreciable increase in cost.

Smoothing the recorded concentration distribution before peak area determination is equally important in reducing the noise-induced error of quantitation. Manual smoothing performed "by eye" is rather ineffective in this regard. Digital techniques can be applied to the problem in two ways, both of which essentially involve "curve fitting" procedures. In the first approach a polynominal of given order is matched to a fixed length sequence of points on the recorded curve. A "least error" criterium, *e.g.*, the least square deviation, is used to determine the coordinates of the smoothed distribution. The other technique, which seems to be more efficient, attempts to fit the theoretically determined concentration profile, *e.g.*, a Gaussian curve, to the recorded distribution[13]. A least error criterium is again used to assess the closeness of the fit.

LASER ILLUMINATION

At very low levels of optical noise, regardless of the way in which they were obtained, electrical noise becomes the decisive factor. It has already been pointed out that a high intensity of the illuminating beam(s) is the most efficient way to offset the consequences of the electrical noise. The energy of the illuminating beams has, however, to be concentrated in a fairly narrow spectral band. The filtering process involved removes a large proportion of the total radiant flux of conventional broad band light sources. In practice there is, therefore, a limit beyond which an increase in beam intensity becomes increasingly difficult.

Tunable lasers appear to be the answer to this problem. It can be expected that the cost of these very recent devices will in the near future decline sufficiently to make them economically competitive. They permit much higher densities of illumination to be obtained as compared with conventional sources. The biggest advantage would be

obtained in the fluorescent mode, where increases in sensitivity of an order of magnitude or more could be expected.

Laser-induced fluorescence offers still another advantage. The laser can be operated so as to emit very short pulses of radiation with extremely high energy densities. If used in conjunction with equally fast photodetectors and associated electronic circuitry, the time constants of the fluorescent radiation could provide sensitivities and selectivities which can today be obtained only by mass spectrometry and similar methods. Micromethods[14,15] recently developed seem already to point in this direction.

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