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DUAL-WAVELENGTH UV-ABSORPTION DETECTION IN CAPILLARY ISOTACHOPHORESIS

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SUMMARY

Instrumentation for dual-wavelength UV-absorption detection in isotachopheresis is described and evaluated. Computerized signal storage and processing allow data reduction on the basis of the ratio of absorption at any two of the wavelengths 206, 254, 289 and 340 nm. The purity of UV-absorbing spikes or zones is verified by plotting the ratio *versus* time, the ratio *versus* one wavelength or one wavelength *versus* the other. The method is illustrated with the analysis of a nucleotide extract of eggs of *Nassarius reticularis*.

INTRODUCTION

In capillary isotachopheresis, the detection limit of a universal (a.c. conductivity or potential gradient) detector is directly proportional¹ to the concentration of the leading electrolyte (*ca.* 0.01 *M*) and the volume of the detector cell (2-100 nl). UV-absorption detection makes it possible to detect zone volumes smaller than the detector cell volume, provided that certain precautions are taken, such as the addition of non-UV absorbing spacers². In this instance, as in UV detection in liquid chromatography, the detection limit also depends on the properties of the sample components such as the molar absorptivity, on interfering substances and on detector noise. The choice of wavelength is an important parameter for decreasing the detection limit. Whereas in isotachopheresis mainly fixed-wavelength detectors (h.f.-excited plasma sources) are used, in liquid chromatography continuously variable UV detection is also applied. This makes it possible to optimize the detection by scanning peaks of interest at stopped flow. Fast scanning detectors, which permit the measurement of absorbance ratios at two wavelengths, are also available. It will take some time for these types of detectors to be introduced commercially for isotachopheresis. The advantages of multiple wavelength detection in isotachopheresis were recognized earlier^{3,4}.

Vacík and Everaerts⁵ used a modified UV spectrophotometer to measure the UV spectrum of an isotachopheretically migrating steady-state mixed zone. The properties of currently available fixed-wavelength detectors for isotachopheresis and liquid chromatography are summarized in Table I.

TABLE I

TYPICAL SPECIFICATIONS OF COMMERCIALY AVAILABLE FIXED-WAVELENGTH UV DETECTORS FOR ISOTACHOPHORESIS (ITP) AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The values in parentheses refer to our home-made ITP equipment¹.

<i>Parameter</i>	<i>ITP</i>	<i>HPLC</i>
Light source	h.f.-excited plasma	Low-pressure lamp
Filter band width	ca. 25 nm	1–10 nm
Cell volume	40–100 nl (2–6 nl)	2–8 μ l
Slit diameter	0.3–0.5 mm (0.1–0.2 mm)	2–5 mm
Path length	0.5 mm (0.2 mm)	0.5–10 mm
Detector	Photodiode	Photomultiplier
Noise	0.0004 a.u.	0.00001 a.u.

In the adaption of UV detectors for isotachopheresis more attention has been paid to the cell volume than to the noise level, mainly because in most instances the signal amplitude is not used for quantitation purposes. This also explains why logarithmic conversion of transmission to absorbance units is not yet standard in isotachopheresis. The use of the so-called UV-spike method and the introduction of zone electrophoresis in capillaries⁶, however, will necessitate both logarithmic conversion and a lower noise level. In spite of the fact that attempts at miniaturization in liquid chromatography have led to detector cell volumes down to 0.3 μ l, significant differences in detector cell dimensions of the two techniques remain.

We have constructed a detector cell for dual-wavelength detection in isotachopheresis⁷ to be used with currently available h.f.-excited plasma lamps (mercury or iodine) and UV-sensitive photodiode detectors. Combinations of filters and plasma lamps allow detection at any two of the wavelengths 206, 254, 280 and 340 nm simultaneously in a 3.5-nl cell volume with an acceptable noise level.

INSTRUMENTAL

The construction of the UV cell is shown in Fig. 1. The PTFE capillary (I.D. 0.2 mm) is pulled through a 0.4-mm hole in a brass cylinder. Perpendicular to this hole, four holes are drilled equiplanar at right-angles. These holes have I.D. 0.15 mm and act as slits for UV detection. Four quartz rods, mounted in brass holders and fixed by a screw, approach the central hole as close as possible to minimize the loss of UV light. The UV sources and detectors were connected at the ends of the quartz rods. Standard components¹ were used to complete the instrument.

The detector signals were monitored by a Puzzle (E. Steiner, Vienna, Austria) microprocessor system equipped with two analogue-to-digital converters (ADC) operated at either 8- or 12-bits resolution. Signal output with two 12-bit digital-to-analogue converters (DAC) was recorded with a Type BD41 potentiometric *X-t* recorder (Kipp, Delft, The Netherlands) or a Type PM8120 *X-Y* recorder (Philips, Eindhoven, The Netherlands). The computer programs for signal evaluation were written in BASIC whereas the sampling frequency was in the range 10–59 Hz.

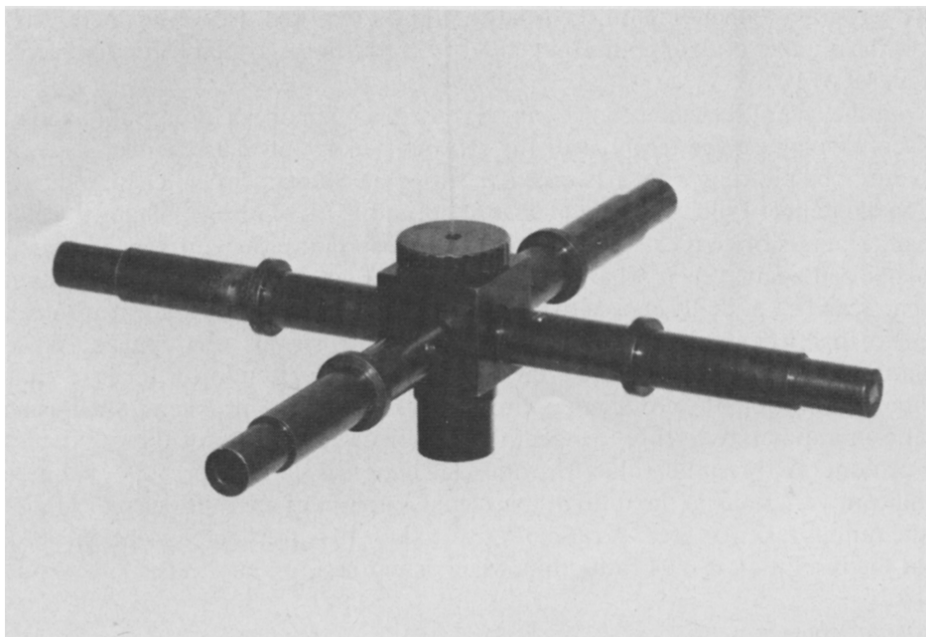


Fig. 1. Construction of the unit for dual-wavelength UV detection in isotachopheresis. Perpendicular to the vertically mounted PTFE capillary, four quartz rods in brass holders are mounted equiplanar.

The operational systems, listed in Table I, were made from analytical-reagent grade chemicals purchased from either Merck (Darmstadt, G.F.R.) or Sigma (St. Louis, MO, U.S.A.). The nucleotides were purchased from Boehringer (Mannheim, G.F.R.).

RESULTS AND DISCUSSION

Signal evaluation

The mutual interference of the two UV light beams perpendicular to one another was determined with detection at 254 nm for both channels, with the filter placed at the detector side. It amounted to less than 1%. This interference was completely eliminated when two different filters were placed before each detector. The noise level of each channel was measured with the DACs at 12 bits (0.025%) resolution. As the 1-Hz region of the noise spectrum of the detector signal is most important in isotachopheresis (with respect to the detector response time required), the amplitude of both detector signals was measured ten times at 1-sec intervals. From these values the average baseline (offset) and noise were calculated. The average noise level was *ca.* 0.1% (0.0004 a.u.) for 206, 254 and 280 nm.

The detector signals were then continuously monitored at 12 bits, 2 Hz, where the baseline values were updated for drift. If the signal-to-noise ratio of one channel exceeds 4, signal storage will commence. Now the full-scale resolution was 8 bits (0.4%), so that the detector noise was filtered out. Baseline offset correction was applied simultaneously. The sampling frequency could be chosen up to 59 Hz in the

BASIC program, depending on the time resolution required. However, there was a limit to the number of data points that could be stored in the available random access memory (RAM).

At the end of the run a choice can be made from a number of output facilities: (a) visual display on the terminal of the ratio of absorption plus channel 1 of the entire run; (b) plotting with a two-pen recorder of channel 1 plus channel 2; (c) plotting of channel 1 plus the ratio of channels 1 and 2; (d) plotting of channel 1 plus those data points of channel 1 that comply with a certain ratio. Further, all signals can be plotted against each other with an X-Y recorder. In all instances the output frequency can be optimally chosen to match the response of the recording instrument.

No logarithmic conversion of the transmission signals was applied. When calculating the UV ratio of a spike, the concentration distribution will cause some non-linearity above 50% absorption. However, this poses no problems when using the ratio in a qualitative sense for identification or determination of the purity of a spike or zone. A threshold value for ratio calculation is chosen for both channels. Insignificant variations in the ratio at low signal amplitudes are thus deleted. In that case the ratio is taken as zero. A ratio of greater than 10 is considered to be off-scale, so that the resolution is 0.04 ratio units when using the DAC at 8 bits.

Selection of zones

Fig. 2 shows the analysis at pH 3.9 (Table II) of a standard mixture of nucleotides detected simultaneously at (a) 254 and (b) 280 nm. It is known from the literature^{3,4,7} that the different classes of nucleotides can be characterized by a certain ratio of absorption at these two wavelengths. This ratio is more specific for a particular class of nucleotide than just the absorption at any of the two wavelengths. A ratio plot (Fig. 2c) illustrates this. The importance of dual-wavelength detection in verifying the purity of zones is also shown.

Whereas detection at one wavelength may suggest a pure zone, an interference will be detected only at the other wavelength. If no choice can be made as to which wavelength is best for a certain class of compounds, such as nucleotides, a ratio plot includes the information of both wavelengths. Quantitation by measuring zone lengths can also be applied to the ratio plot. Further data reduction by the micro computer is possible. The entire dual-wavelength isotachopherogram can be plotted from the memory, but it can also be limited to those zones which comply with a certain ratio. This is illustrated in Fig. 2, in which the original 254-nm isotachopherogram is shown together with a reduced isotachopherogram (Fig. 2d), where only the zones with a ratio of 3.0 are seen. For this facility, a ratio window for recognition has to be used. The width of this window (20% in this instance) must be greater than the variation of the ratio of the zones to be selected. Because of the sequence of the zones, the qualitative information from the time axis is more pronounced. Alternatively, the output can be limited to zones with a ratio above or below (Fig. 2e) a pre-set value. A threshold value for ratio calculation of 5% absorption was used in this instance.

The method of selection of zones on the basis of the UV ratio was applied to the analysis of an extract of nucleotides from sea snail (*Nassarius reticularis*) eggs⁴. Fig. 3a and b show the isotachopherograms at 254 and 280 nm, respectively. Positive identification is obtained from the ratio plot (Fig. 3c), where the plateau value of the ratio corresponds to the value in the standard mixture. Therefore, this ratio can be

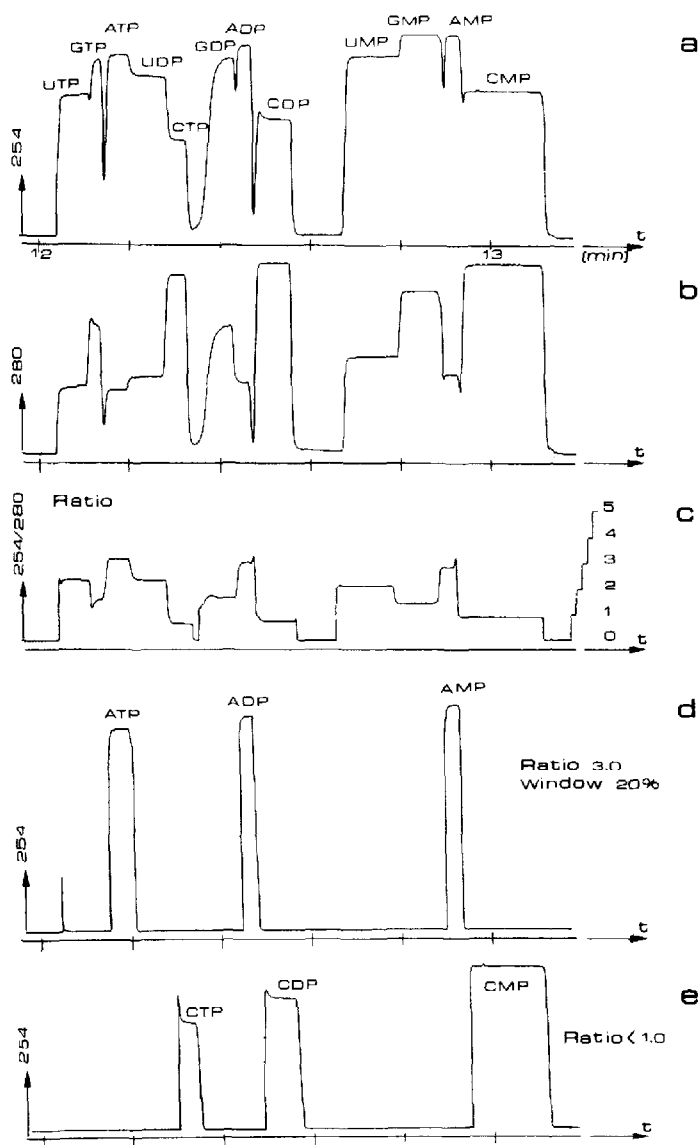


Fig. 2. Analysis at pH 3.9 (Table II) of a standard mixture of nucleotides, detected simultaneously at (a) 254 nm and (b) 280 nm. The 254/280 nm absorption ratio (c) can be plotted from the computer memory. Each class of nucleotides is characterized by a distinct ratio: cytidine *ca.* 0.8, guanosine *ca.* 1.5, uridine *ca.* 2.4 and adenosine *ca.* 3.0. Selected output of the isotachopherogram at 254 nm is possible on the basis of this ratio. A ratio of 0.3 with a 20% window will select the zones of adenosine nucleotides (d). In this sample only the cytidine nucleotides have a ratio smaller than 1 (e). The threshold value for ratio calculation was 5%.

used as a third method of identification, together with the conductivity or potential gradient trace (not shown) and the absorption at any wavelength. In this example there is no preference for any of the two wavelengths, in terms of the height of the plateau values. For the adenosine nucleotides 254 nm seems to be the best choice, but

TABLE II
OPERATIONAL SYSTEMS FOR ISOTACHOPHORETIC ANALYSES OF ANIONS

The current was 25 μ A in a PTFE capillary (I.D. 0.2 mm). PVA = poly(vinyl alcohol), Mowiol (Hoechst, Frankfurt, G.F.R.); CTAB = cetyltrimethylammonium bromide (Merck, Darmstadt, G.F.R.); HEC = hydroxyethylcellulose (Polysciences, Warrington, PA, U.S.A.); MES = 2-morpholinoethanesulphonic acid.

Parameter	pH 3.9	pH 6.0
Leading ion	Chloride	Chloride
Concentration	0.01 M	0.01 M
Counter ion	β -Alanine	Histidine
Additive	0.05% PVA 0.02 mM CTAB	0.05% PVA 0.2% HEC
Terminating ion	Caproate	MES
Concentration	ca. 0.005 M	ca. 0.005 M

for the cytidine nucleotides clearly 280 nm is to be preferred. For isotachophoretic zones smaller than the detector cell volume, the plateau value of the absorption will decrease. If, however, such a UV spike consists of only one UV-absorbing compound, the concentration distribution will not affect the UV absorbance ratio. If this ratio is not constant during the detection of a UV spike, a UV-absorbing interference can be suspected. In Fig. 3c this is probably the case with the CDP spike, in spite of the fact that both the 254- and the 280-nm spike do not indicate an impurity (no shoulder). The use of the UV ratio to verify the purity of UV spikes was therefore investigated further.

Purity of spikes or zones

Fig. 4 shows an example of the different methods for signal representation by the method described. From the 254-nm trace of a UV spike (Fig. 4a) no impurity is suspected, probably not even from the second differential, a method used to detect impurities in chromatographic peaks. In the 280-nm trace (Fig. 4b) a shoulder is present, although 280 nm is, in this example, not the optimal wavelength with respect to response. A ratio plot (254/280 nm) from the computer memory (Fig. 4c), however, indicates three compounds. Each of these is characterized by a certain absorption ratio at the wavelengths chosen and can therefore be represented by a point in the 254 versus 280 nm absorption vector space. This is illustrated in Fig. 4d, where the 280-nm absorption is plotted against the 254-nm absorption with an X-Y recorder. This figure gives only a qualitative indication of the purity of a spike or zone, as time information is obscured in this representation. A pure UV spike or zone would have given a straight line from the origin to the corresponding point in the 280 versus 254 nm vector space. The slope of that line would correspond to the reciprocal value of the 254/280 nm ratio. Also, the 254/280 nm absorption ratio can be plotted against the 254-nm absorption on an X-Y recorder. Here also the three compounds with their respective ratios can be recognized (Fig. 4e). In this example compound 2 was deliberately added, whereas compounds 1 and 3 were present as impurities between the spacers.

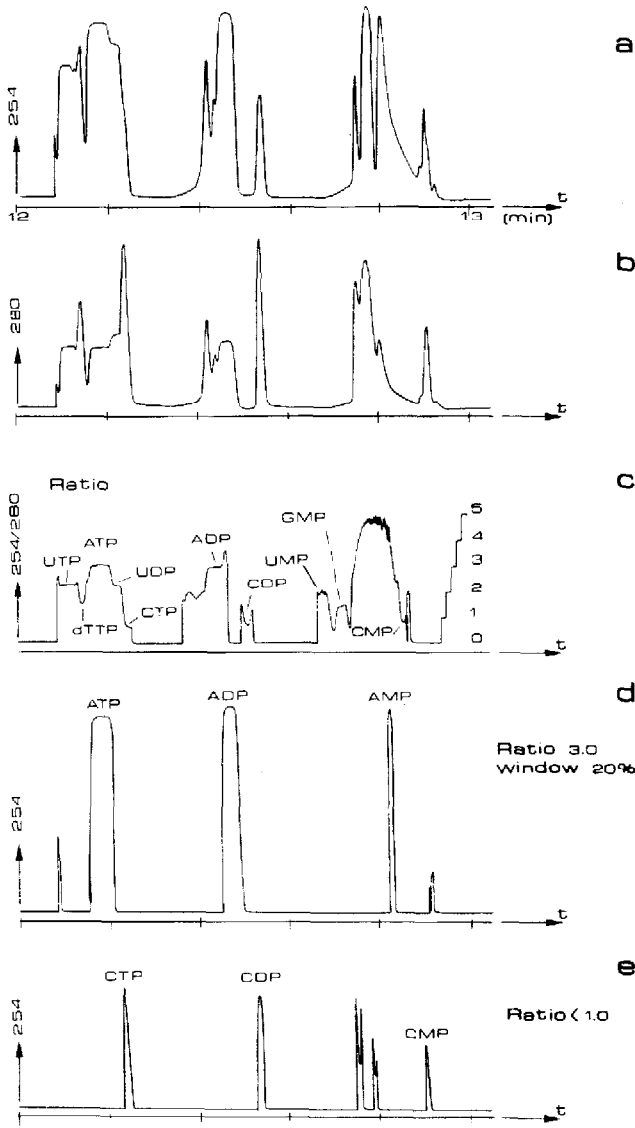


Fig. 3. Analysis at pH 3.9 of a methanol (50%) extract of sea snail (*Nassarius reticularis*) eggs⁴ detected at (a) 254 nm and (b) 280 nm. Several nucleotides were identified by the plateau value of the 254/280 nm absorption ratio (c). For small zones/spikes such as CDP, the ratio indicates possible interfering substances. Adenosine nucleotides, for example, can be selected with a ratio of 3.0 and a 20% window (d). In addition to the cytidine nucleotides, other minor constituents also comply with a ratio smaller than 1 (e).

Two standard mixtures of UV- and non-UV-absorbing compounds were prepared and analysed in different proportions in an operational system at pH 6.0. In an analysis of the non-UV-absorbing compounds only, a number of UV-absorbing impurities can be seen (Fig. 5a). These originate from either the sample or the operational system. The ratio plot indicates that between some of the spacers more than one impurity is present. When in addition to the spacers a number of UV-absorbing

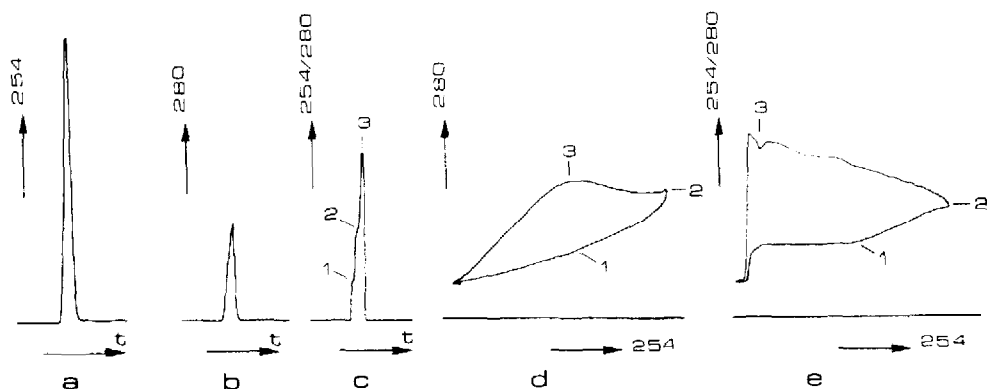


Fig. 4. Analysis of a UV spike of 10 ng of sulphaniolate (2) with acetate and glutamate as spacers at pH 6.0 (Table II). The relative effective mobility difference between the spacers was 22%. Detection was performed at (a) 254 nm and (b) 280 nm simultaneously. The 280-nm trace indicates one impurity, whereas the UV-absorption ratio (c) indicates two impurities (1 and 3). Each of the constituents of the spike can be characterized by a point in the 280 nm versus 254 nm vector space (d) or in the 254/280 nm ratio versus 254 nm plot (e). See text for further explanation.

compounds are injected (Fig. 5b), the contribution of the impurities can only be neglected for peaks 2, 4 and 8, as can be seen from the corresponding ratio plot. Peaks 6 and 10 contain major impurities. When a plateau value in the absorption of the compounds is reached (Fig. 5c), the amplitude of the ratio signal of the pure zones (4 and 8) does not differ from the value of the spikes (Fig. 5b). Quantitation by zone-length measurement in this instance is more reliable when using the ratio plot, as illustrated.

In Fig. 5d, the 280 nm versus 254 nm plots of the zones in Fig. 5c are shown. Zones 6 and 10 contain most impurities, as can be seen from the deviation from a straight line. Zones 2, 4, and 8 are relatively pure.

The number of interfering substances when using the so-called UV spike method can be decreased by decreasing the relative effective mobility difference between the spacers. Indeed, this relative difference was smallest (13%) for peaks 4 and 8 in Fig. 5, although minor impurities were present between all spacers in this standard mixture.

CONCLUSIONS

Dual-wavelength detection with ratio measurement is a useful method for determining the purity of UV spikes or zones in isotachopheresis, especially when developing a UV spike method^{2,8} to be used in a complicated matrix. It has obvious advantages as an additional method of identification of UV-absorbing compounds and mixed zones in isotachopherograms with many UV-absorbing zones. The use of computerized signal processing, however, is imperative. In addition to the 280/254 nm combination mentioned, the use of 206 and 280 nm is especially useful for the identification of peptides and proteins^{9,10}, as these wavelengths give information on the number of peptide bonds and UV-absorbing amino acids, respectively.

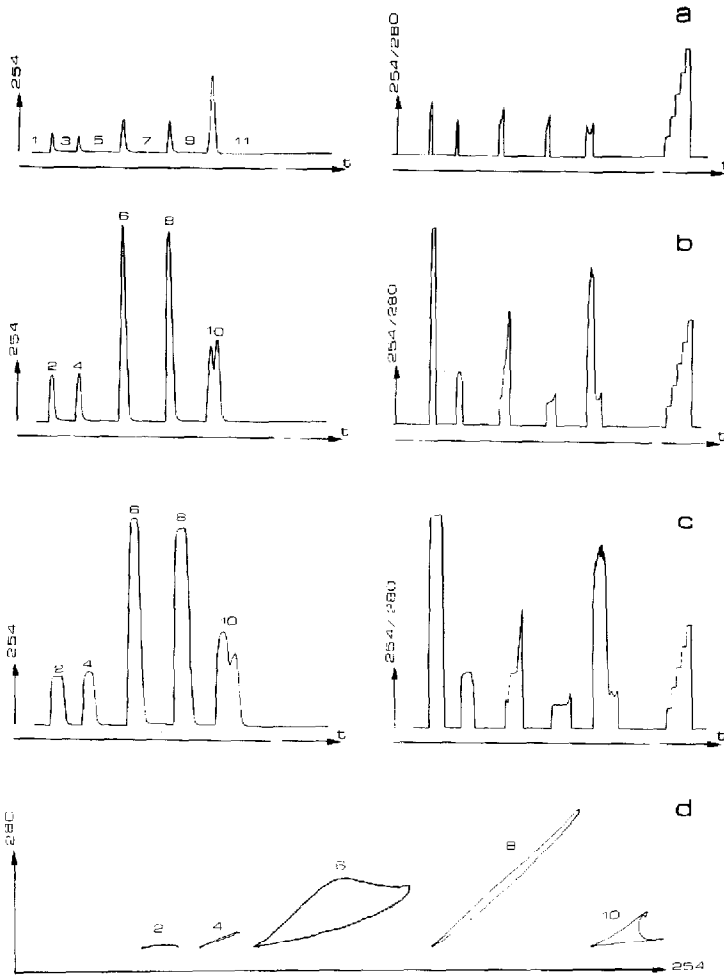


Fig. 5. Analysis of ca. 100 ng each of the following non-UV-absorbing substances at pH 6.0 (a): 1 = Formate; 3 = glucarate; 5 = acetate; 7 = butyrate; 9 = glutamate containing minor impurities; 11 = MES. This mixture was also analysed together with 2 = pyrazole-3,5-dicarboxylate, 4 = phthalate, 6 = sulphanillate, 8 = *p*-aminobenzoate and 10 = hippurate. Of these, (b) 10 ng and (c) 30 ng of each were injected. The peaks 2, 4, 6, 8 and 10 from the latter are also represented in the 280 nm versus 254 nm plot (d).

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