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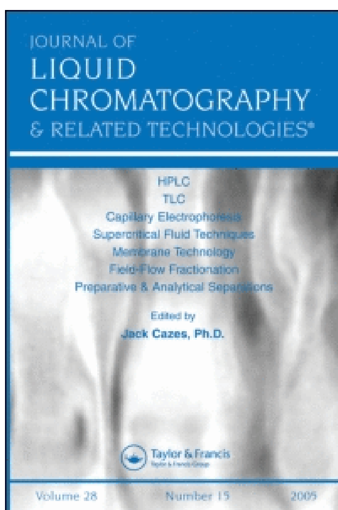

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GRADIENT ELUTION OF BIOGENIC AMINES AND DERIVATIVES IN REVERSED PHASE ION-PAIR PARTITION CHROMATOGRAPHY WITH ELECTROCHEMICAL AND FLUOROMETRIC DETECTION

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SUMMARY

The compatibility of gradient elution and reversed phase ion-pair partition systems combined with electrochemical and fluorometric detection has been investigated. The phase system consisting of buffers with perchlorate counter-ions as mobile phases and tri-n-butylphosphate as stationary phase allows the use of pH and counter-ion gradients. It appeared that (i) use of gradients is time saving and favourable with respect to detection limits and (ii) dual electrode detection may offer a solution to the problem of gradient-induced baseline shifting in electrochemical

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detection. Native fluorometric detection allows the monitoring on nanogram level in gradient runs. The method described is applied to biological samples, i.e. rat brain tissue (striatum).

INTRODUCTION

The determination of biogenic amines, precursors and metabolites with HPLC techniques combined with electrochemical detection has become increasingly important, since the selectivity and the sensitivity of this combination allows the separation and detection of a number of such compounds in the subnanogram level (1-3).The compounds of interest often have diverse characteristics in the now commonly used reversed phase systems. Dependent on the solutes studied, run times may be lengthy, which hampers the application for routine analysis requiring short run times and a high throughput of samples. Besides, high values of the capacity ratios results in unfavourable detection limits, as can be concluded from equation (1).

$$(1) \quad c^{m}_{i,max} = \frac{m_i \sqrt{N_i}}{\sqrt{2\pi}\,(1+k')V_m}$$

where $c^{m}_{i,max}$ is the maximum outlet concentration of compound i, m_i is the injected amount, N_i is the theoretical plate number, k' is the capacity ratio and V_m is the volume of the mobile phase in the column.

There are several ways to speed up analysis. Application of the so-called High Speed systems using smaller particle sizes resulting in highly efficient columns allows the use of shorter columns with smaller void volumes V_m, which in practice is partly counteracted by the commonly increased inner diameters, necessary for a reasonable loading capacity of the column. However, introduction of larger sample volumes is limited, especially in isocratic runs (4,5).

Another way to speed up analysis is to make use of gradients during the chromatographic run. The effect of gradients on the detection limit is twofold: (i) improvement of the

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maximum outlet concentration, caused by peak compression during the run, which is favourable and (ii) increase of the baseline shortterm noise and longterm noise (shift), which is unfavourable for the detection limits. Besides, with respect to analysis time one should realize that gradient elution demands a regeneration time for the column, which must be taken into account for the total analysis time.

Increase of the baseline noise is a severe drawback of the use of gradients with electrochemical detection, which is caused by the high sensitivity of electrochemical detectors towards changes in the composition of the mobile phase especially with respect to pH and ionic strength and only to a slight extent (6-8) with respect to the modifier content.For the reasons mentioned above, most workers prefer isocratic conditions for their analyses, especially if trace level analysis is required.However, there are some interesting possibilities to correct for this baseline shift in gradient HPLC: (i) correction by means of the method of background subtraction, which has some drawbacks, since it demands a highly reproducible gradient system and a system to store blank runs, while increasing the effective runtime, because of the need for blank runs, (ii) the use of a dummy system with identical characteristics as the measuring system (9) and (iii) the use of dual detection systems in which the two working electrodes are placed in series towards the flow direction (6).

We studied the applicability of the latter mode of correction. Changes of current occurring during the chromatographic runs can be caused by several processes :

$$(2) \quad \Delta i_{tot} = \Delta i_{f,analyte} + \Delta i_{c+f,grad}$$

with i_{tot} is the total current, $i_{f,analyte}$ is the faradaic component caused by the analyte and $i_{c+f,grad}$ is the component caused by the gradient, which component can include both faradaic and charging currents. The problem is to distinguish between $i_{f,analyte}$ and $i_{c+f,grad}$. This can be achieved by applying the same potential to both working

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electrodes (6,10) and subtracting the signal of the downstream detector from the signal of the upstream detector. In this way the downstream electrode or so-called shift-detector, is used to follow changes in the mobile phase.

Two types of electrochemical detectors can be taken into account, viz. the coulometric and amperometric detectors. Supposing that Δi_{grad} is only caused by charging current and that both cells have similar electrochemical characteristics, this contribution to the total current i_{tot} is the same for the upstream and downstream detector, and the differential signal is therefore corrected for gradient effects. In practice Δi_{grad} also includes a faradaic current resulting in only a partial correction for dual coulometric cells but in principle complete correction for amperometric types (6,8). However, in dual coulometric detection systems $i_{f,analyte}$ at the downstream detector is negligible and so in the differential signal information concerning the analyte is not attenuated.

It is also possible to apply different potentials to the working electrodes whereby the shift detector has a potential lower than the half-wave potential of the compounds of interest assuming that the shift characteristics are similar at different potentials.

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EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a high-pressure gradient system (SP 3500B, Spectra Physics, Santa Clara, CA, U.S.A.) equipped with thermostatted eluent reservoirs, a thermostatted stainless steel column (100 x 3 mm I.D.) and different detection systems. The amperometric detection system consisted of a so-called wall-jet detector cell-unit (E.D.T., London, U.K.) coupled with a potentiostat (E-230, Bruker, Karlsruhe, G.F.R.). The detector cell was modified

by replacement of the Ag/AgCl reference electrode by a home-made saturated calomel electrode. The (dual) coulometric detection system including a guard cell, placed between the dynamic mixer of the gradient system and the injector, consisted of a dual detector cell-unit coupled with a dual potentiostat (Coulochem, Model 5100A, ESA, Bedford, MA, U.S.A.). The fluorometric detector was a double monochromator type with 150 Watt Xe-source (SFM 23 LC, Kontron, Zurich, Switzerland). Chromatograms were registered with flat-bed recorders (BD-8, Kipp & Zn., Delft, The Netherlands).

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Chemicals and Materials

The compounds used as chromatographic reference substances are listed in Table I. All other chemicals and solvents were of analytical or reagent grade and were used without further purification except for water which was purified by a Milli-Q Water Purification System (Millipore, Bedford, MA, U.S.A.). The chromatographic support material was Nucleosil C-8, 5 μm (Macherey-Nagel, Duren, G.F.R.). Columns were packed by means of a slurry technique described elsewhere (11).

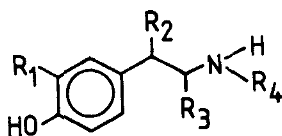
The constituents of the mobile phase were saturated with tri-n-butylphosphate (Aldrich, Milwaukee, WI, U.S.A.) before use.

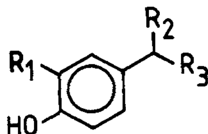
Columns were loaded with tri-n-butylphosphate (TBP) in situ as described elsewhere (4,5). Thus a liquid-liquid reversed phase system was built up with TBP saturated buffers as mobile phases and TBP as stationary phase.

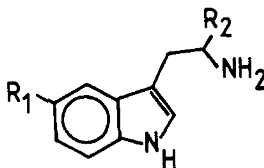
Chromatography

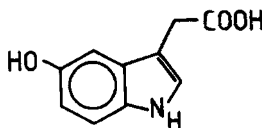
The capacity ratio k' was determined from its retention time t_{Ri} and the retention time of an unretained compound t_{RO}, for which potassium iodide was used.

TABLE I STRUCTURAL TYPES OF INTEREST









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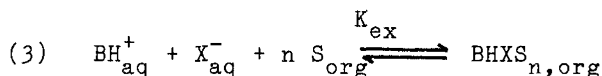
THEORETICAL

The compounds of interest can be divided in amines, acids, amino acids and neutral compounds (see Table I). Each group has its own distribution characteristics between the two liquid phases. Taking into account a possible ion-pair dissociation in the organic phase (S), the distribution of a protonated amine (BH) with perchlorate (X) as counter-ion can be described as follows (4,5) :



$$(3) \quad BH^+_{aq} + X^-_{aq} + n\ S_{org} \overset{K_{ex}}{\rightleftharpoons} BHXS_{n,org}$$

$$(4) \quad BHXS_{n,org} \underset{}{\overset{K_{diss}}{\rightleftharpoons}} BHS^{+}_{n,org} + X^{-}_{org}$$

From these equations an expression can be derived describing the distribution of amines :

$$(5) \quad D_{amine} = K_{ex} . [X^{-}]_{aq} . [S]^{n}_{org} \left(1 + \frac{K_{diss}}{[X^{-}]_{org}} \right)$$

Equation (6) gives the distribution for un acid HA :

$$(6) \quad D_{acid} = \frac{K_{HA}}{1 + \frac{K_a}{[H+]}}$$

where K_{HA} is the partition coefficient of the undissociated acid and K_a the acid dissociation constant. Combining equations (5) and (6) an expression for the distribution of an amino acid ion-pair can be derived :

$$(7) \quad D_{amino\ acid} = K_{ex} [X^{-}] [S]^{n}_{org} \left(1 + \frac{K_{diss}}{[X^{-}]_{org}}\right) \left(1 + \frac{K_a}{[H^{+}]}\right)^{-1}$$

where K is the acid dissociation constant of the carboxylic group of the amino acid.
From equations (5), (6) and (7) it can be concluded that, depending on the physico-chemical properties, the capacity ratios can be influenced by the counter-ion concentration and/or the pH of the mobile phase. The retention of neutral compounds can be influenced only by adding an organic modifier to the mobile phase.

RESULTS AND DISCUSSION

Chromatography

In figure 1 a typical chromatogram of a test mixture of the 13 reference substances under isocratic conditions is given. Although the compounds are well separated, it will be clear that this type of chromatography is rather time-consuming and that for the more retained compounds the dilution during the chromatographic process is considerable. The analysis time can be shortened by increasing the flow-rate. However,

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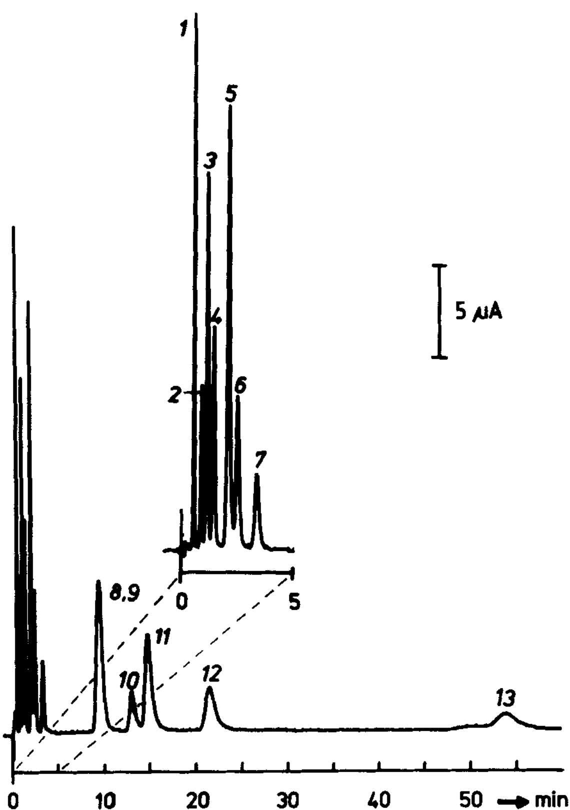

Fig. 1. Separation of the test mixture under isocratic conditions. Perchlorate concentration 0.2 m, pH 2.15, temperature 300 K, coulometric detection +700 mV.
Compounds: 1. MOPEG; 2. A; 3. NA; 4. DOPA; 5. VMA; 6. DA; 7. TYR; 8. 5-HTP; 9. HVA; 10. DOPAC; 11. 5-HT; 12.TRP; 13. 5-HIAA.
Injected amounts: 150 - 520 pmole, except for 5-HIAA: 1270 pmole.

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because of pressure limitations, this can only be done to a certain extent and even leads to less favourable detection limits (see eq.(1)).

Application of gradients is a better way to solve this problem, although a regeneration time for the column is necessary. In principle there are three basic parameters to influence the retention, i.e. the pH, the counter-ion

concentration and the modifier content of the mobile phase (12,13). Obviously the use of modifier gradients can give raise to stability problems because the stationary phase can be stripped off. Therefore, we studied only the pH and counter-ion gradients.

In order to get insight into the retention behaviour of the compounds under gradient conditions and to optimize the gradient, the influence of the counter-ion concentration and the pH of the mobile phase was investigated separately.

In figure 2 the dependence of the capacity ratios on the pH is given. According to equations (6) and (7) the slope of the log k' vs. pH plot amounts to about -1 for pH values above the pK value of the acids and amino acids.

In figure 3a,b the relation between the capacity ratios and the perchlorate concentration in the mobile phase is given for pH 2.25 and 6.00. As can be derived from equations (5) and (7) the capacity ratios of the amines and the amino acids should be proportional to the counter-ion concentration, which is confirmed by the experimental data. It should be noted thar for amines the same value for the capacity ratio is expected for the same counter-ion concentration at both pH 2.25 and 6.00. However, as is demonstrated by the plots in figure 3, the capacity ratios of the amines slightly increase with raising pH of the mobile phase, a phenomenon that has been observed before (4,5). This indicates that the net effect of decreasing the perchlorate concentration is less than expected, if the pH is raised simultaneously during the gradient. The dashed lines represent the linear interpolation between the initial and final conditions of the optimal gradient used in our experiments. From the slope of these lines it can be concluded that the pH is more effective than the counter-ion gradient. In figure 4, which shows the chromatogram of a 9 minutes linear gradient, this effect declares itself obviously (see peaks 12 and 13). Notice that the concentration of 5-HIAA (peak 12) in the test mixture for the gradient is decreased three-fold with respect to the mixture used in the isocratic run. The time, needed for

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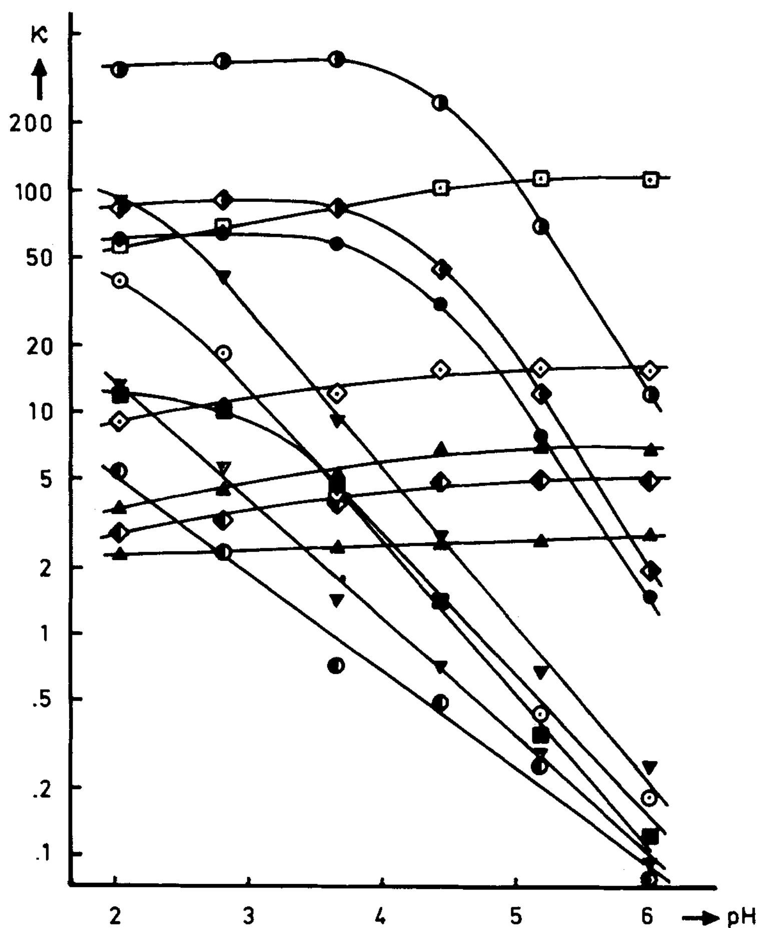

Fig. 2. Influence of the pH of the mobile phase on the capacity ratios.
Perchlorate concentration: 0.10 M, temperature 298 K.
Compounds: ⊡ 5-HT; ◇ DA; ◑ 5-HIAA; △ NA; ◆ A; △ MOPEG; ◆ DOPAC; ● HVA; ▼ TRP; ⊙ 5-HTP; ■ VMA; ▽ TYR; ◐ DOPA.

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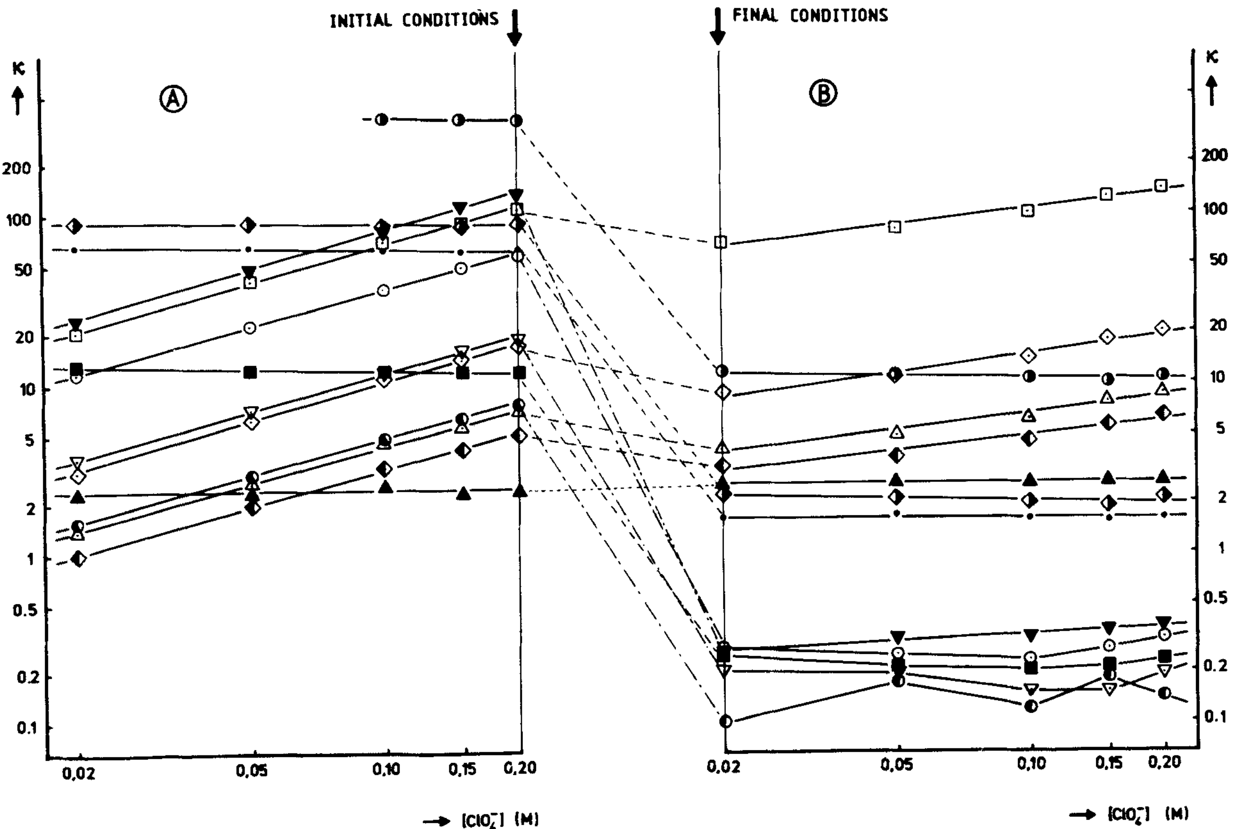

Fig. 3. Influence of the perchlorate concentration in the mobile phase on the capacity ratios at pH 2.25 (A) and pH 6.00 (B).
Temperature 298 K, compounds: see Fig. 2.

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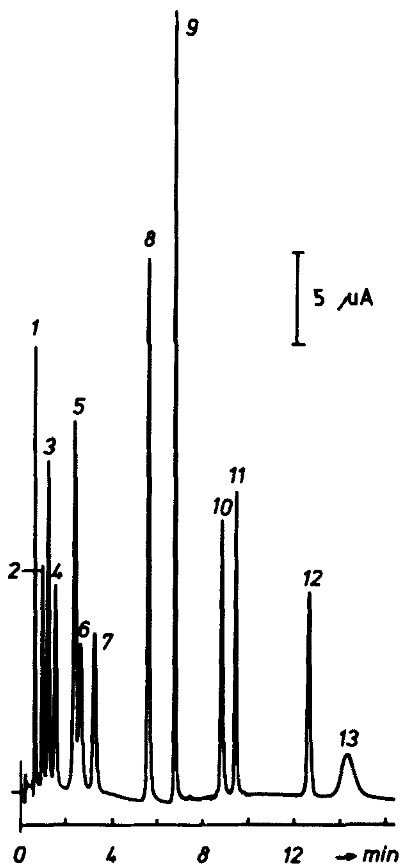

Fig. 4. Gradient elution of the test mixture. Eluent A: perchlorate 0.20 M, pH 2.15. Eluent B: perchlorate 0.02 M, pH 6.00.
Gradient: 9 minutes linear from 0 to 99% B. Temperature 300 K, flow-rate 1.5 ml/min. Coulometric detection +700 mV.
Compounds: 1 - 8 as in Fig. 1; 9. TRP; 10. HVA; 11. DOPAC; 12. 5-HIAA; 13. 5-HT. Injected amounts: 180 - 520 pmole per compound.

re-equilibration, the so-called regeneration time, appeared to be less than 5 minutes resulting in a total analysis time of about 20 minutes.

Influence of the gradient on the detection system

Electrochemical detectors are very sensitive towards changes in the composition of the mobile phase. This effect is clearly demonstrated in figure 5, which shows the chromatogram of a test mixture of the 13 compounds of interest under gradient conditions. Detection was performed by means of an amperometric detection system. It is obvious that the full scale deflection (i.e. 50 nA) can hardly be increased, due to the baseline shift during the run. It is observed that use of a new or recently polished working

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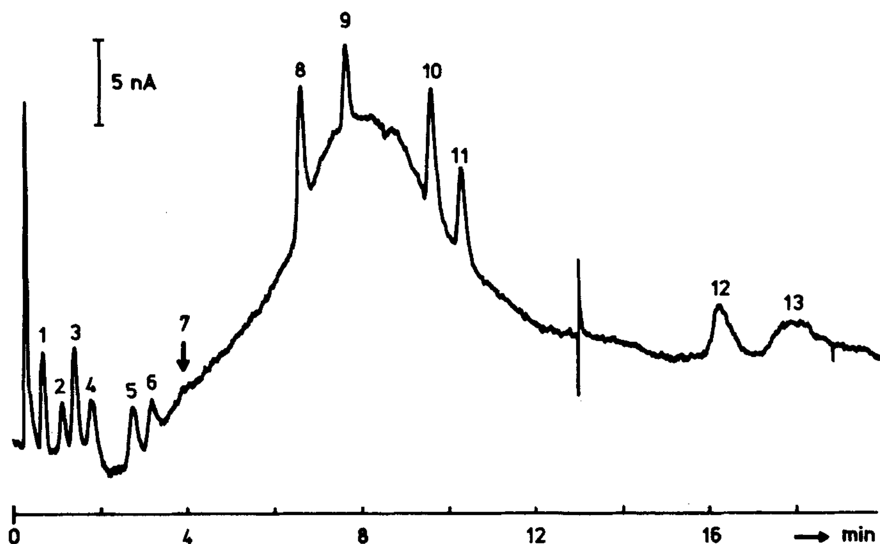

Fig. 5. Amperometric detection of the test mixture after gradient elution.
Applied potential +800 mV vs. SCE, time constant 1 s, flow-rate 1.3 ml/min., temperature 300 K. Compounds: see Fig. 4. Injected amounts: 7.2 - 20.7 pmole per compound. Gradient: see Fig. 4.

electrode resulted in a less prominent shift. However, as a function of time the shift increased.

Next to electrochemical activity the compounds of this study have a native fluorescence, which can be used for detection on subnanogram level (14). Due to the small difference between the excitation and emission wavelength (248 and 318 nm respectively) one should apply a double monochromator system. In figure 6 the chromatogram of the same test mixture with fluorometric detection is given. This kind of

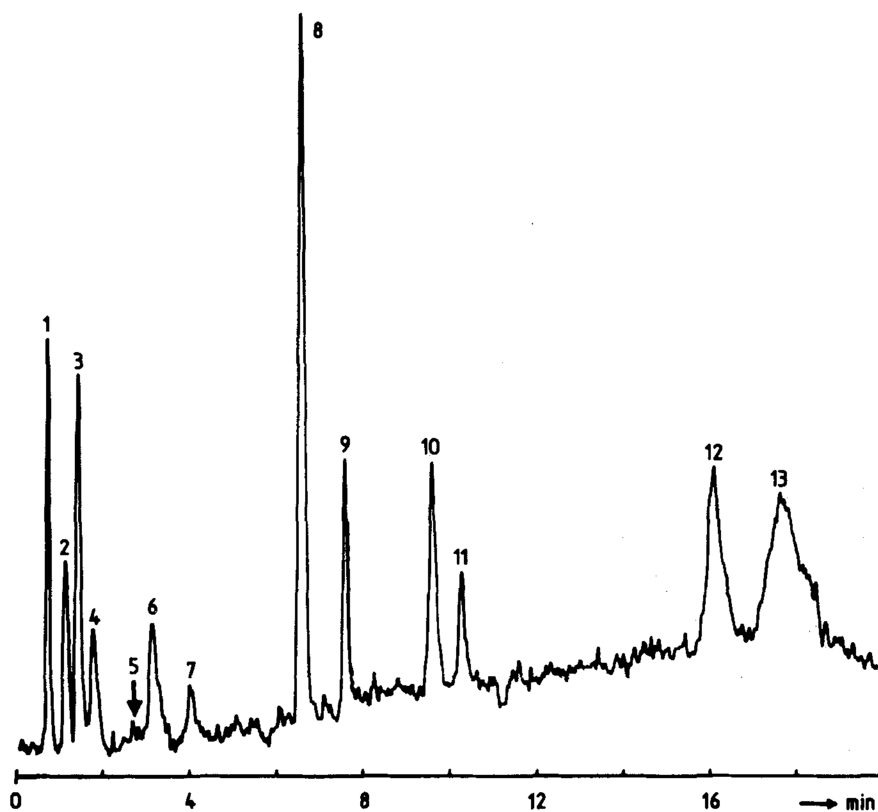

Fig. 6. Fluorometric detection of the test mixture after gradient elution. Excitation wavelength 284 nm, emission wavelength 318 nm.
Conditions: see Fig. 4. Injected amounts: 7.2 - 20.7 pmole per compound.

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detection appears to be less sensitive towards changes in the mobile phase. Besides, the sensitivity for TRP and derivatives is more favourable than with electrochemical detection, which corresponds with data from literature (14). Nevertheless, with fluorometric detection it is not possible to detect all the compounds at lower levels without derivatization (e.g. see VMA). Therefore, we studied the possibilities of a dual electrochemical detector to overcome the gradient induced shift problem.

Figure 7 shows the principle of baseline detection with dual coulometric detection. From trace B (downstream detector) it can be seen that at +700 mV most compounds are converted coulometrically in the upstream detector (trace A). However, the shape of the gradient induced baseline shift is more or less the same for both detectors, which slightly indicates that $\Delta i_{gradient}$ (see eqn. 2) is mainly caused by charging current contributions ($\Delta i_{c,gradient}$), or alternatively that as far as faradaic contributions ($\Delta i_{f,gradient}$) are involved the upstream detector does not work coulometrically. A further indication that $\Delta i_{f,gradient}$ is negligible relative to $\Delta i_{c,gradient}$ comes from the fact that although traces A and B (Fig. 7) have the same shape, the absolute differences are more pronounced for the downstream detector and therefore before subtraction its signal is reduced to 60%. The difference in sensitivity of both cells are caused by differences in activity. It was observed that the yield of conversion for a number of compounds amounts to more than 100% (e.g. MOPEG 260%), which explains the differences in activity for both cells. The peak in trace B which coincides with adrenaline (peak 2) is caused by injection. The differential signal is also corrected for this phenomenon.

The results as shown in figure 7 demonstrate the possibilities of baseline correction by means of the downstream shift detector. However, subtraction leads to an increase of noise (see Fig. 7c) and consequently in the flat parts of trace A subtraction does not improve detectability. It should be emphasized that until now no attempts were made to reduce the noise of the differential signal, which should

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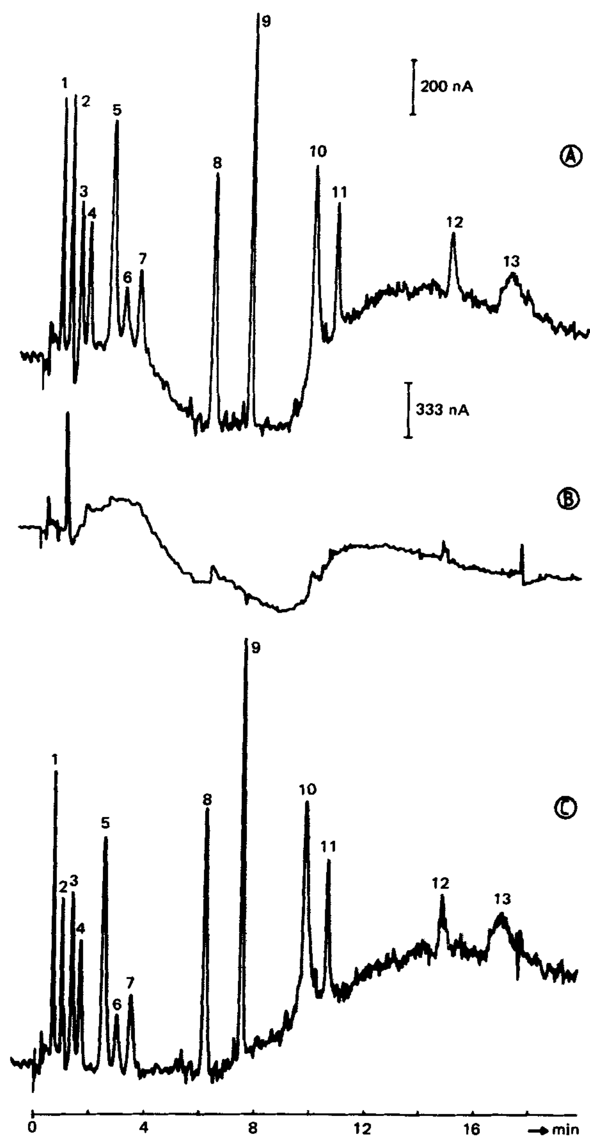

Fig. 7a,b,c Dual coulometric detection of the test mixture after gradient elution. Applied potentials: 700/700 mV. Conditions: see Fig. 4. Injected amounts: 7.2 - 20.7 pmole per compound.
(A) Signal of the upstream detector (2 μA FS)
(B) signal of the downstream detector (3.33 μA FS)
(c) Differential signal (see text).

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be possible, for example by means of "time-delayed subtraction".

The power of the dual coulometric detection system is more clearly shown in figure 8, which shows the chromatogram of a homogenate of rat brain tissue. The sample is pretreated as described elsewhere (15). The quantitative results are summarized in Table II.

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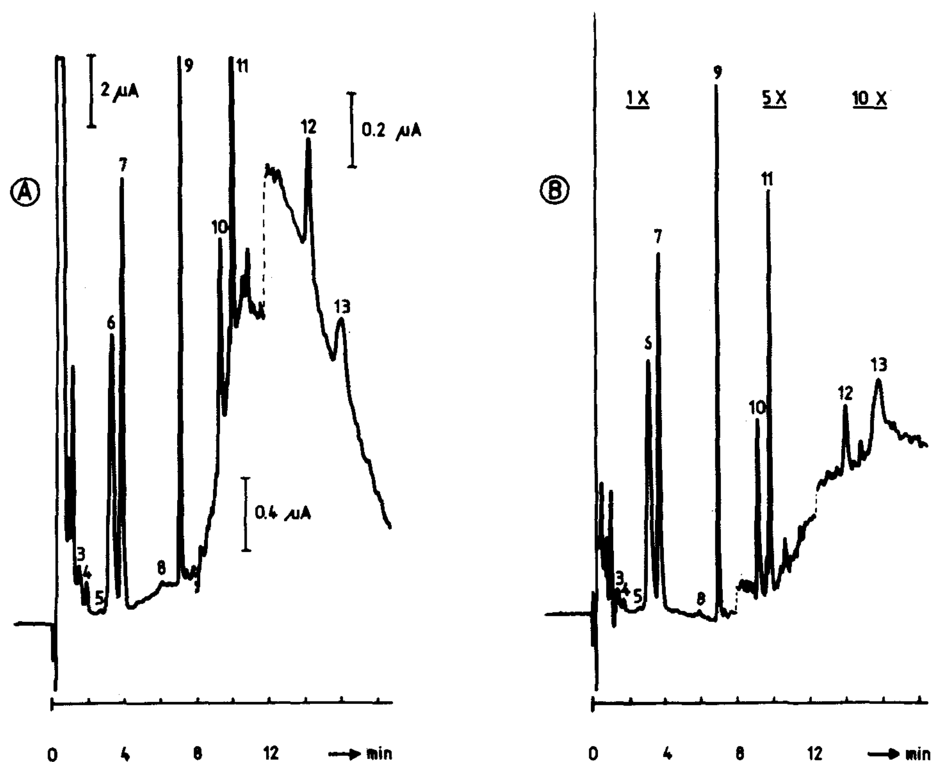

Fig. 8a,b . Analysis of a rat striatum homogenate. Dual coulometric detection: 800/800 mV. Conditions: see Fig. 4, except for flow rate: 1.4 ml/min.
(A) Signal of the upstream detector
(B) Differential signal (see text)
Injection volume: 100 1 corresponding to 4 mg striatal tissue.

TABLE II

Compound	amount injected (pmole)	tissue content (ng/g)
MOPEG	n.d.[1]	n.d.[1]
A	n.d.[1]	n.d.[1]
NA	15.3	650
DOPA	11.4	560
VMA	4.5	220
DA	514	19670
TYR	484	21950
5-HTP	1.9	100
TRP	171	8720
HVA	34.6	1575
DOPAC	124	5190
5-HIAA	15.2	724
5-HT	17.2	750

[1] not detectable

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CONCLUSIONS

Compared to isocratic chromatography the advantage of gradient elution of biogenic amines and their main precursors and metabolites is two-fold: speeding up analysis and lowering detection limits of especially the more retained compounds.

Fluorometric detection is relatively insensitive towards changes of the pH and the ionic strength of the mobile phase and offers detection limits comparable with electrochemical detection.

Electrochemical detectors are rather sensitive towards changes in the mobile phase. Applying the differential coulometric detection principle the gradient induced baseline shift is reduced and consequently the quantification of a number of compounds is facilitated, although noise levels are slightly increased.
Future research will be devoted to noise investigations in order to minimize detection limits.

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