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# OPTIMIZED DETERMINATION OF INDOLE DERIVATIVES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH SELECTIVE DETECTION

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## SUMMARY

A procedure for the optimum separation and determination of indole derivatives was developed, based on a generally applicable algorithm for computer processing of experimental data. Experiments were performed on a Separon SGX $C_{18}$ column with a mobile phase of citrate phosphate buffer (pH 3.1), containing various amounts of ethanol. A tandem combination of an UV-photometric detector at 267 nm and an electrochemical detector with a wall-jett cell was used. The detection limit was $10^{-10}$ g for certain indole derivatives. The optimization by means of the computer program consists of the following steps: display of the dependence of the capacity factors on the ethanol content in the mobile phase, display of the course of the optimization function and construction of separation maps and simulation of chromatograms.

## INTRODUCTION

The analysis of indole derivatives is of some importance. A number of them are growth regulators in plants and microorganisms (auxins). Certain indole compounds, tryptamine derivatives in particular, exhibit psychotropic effects and are monitored primarily in forensic chemistry and toxicology. Indole-type compounds are also important neurotransmitters, their precursors or metabolites.

Reversed-phase systems[1–15], usually $C_{18}$ or $C_8$[9], have been used for separations in high-performance liquid chromatography (HPLC). Separation on adsorption columns or strongly acidic cation exchangers[3] has been less commonly used. Alcohol mixtures (methanol, ethanol) or mixtures of acetonitrile with buffers, acids or occasionally with an ion-pairing agent have usually serve as the mobile phases. The optimization of the mobile-phase composition for the separation of indole compounds has been studied in detail in a number of papers[6,9,14].

UV-photometric[3,6,10,11,15], electrochemical[1–7,9,12,13] or fluorometric[1,3,8,11,15] detectors have been used. Absolute identification by mass spectrometry[8,15] or controls based on the ratio of responses of fluorometric and electrochemical detectors have been used for the identification of peaks of individual compounds[1].

It was the aim of the present work to propose and verify a rapid procedure

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for optimization of the separation and detection conditions for indole derivatives. The selectivity of the electrochemical detector was used in order to obtain an efficient separation.

## EXPERIMENTAL

### *Chemicals*

The analytical standards of indole substances were from Sigma (St. Louis, MO, U.S.A.), except 4-phosphoryloxy-N,N-dimethyltryptamine (psilocybin) (Sandoz, Basle, Switzerland). Other chemicals (Lachema, Brno, Czechoslovakia) were of analytical grade.

### *Instrumentation*

A Model 3 B high-pressure pump, a Model 7105 injection valve and a LC-75 spectrophotometric detector (all from Perkin-Elmer, Norwalk, CT, U.S.A.) were used for chromatography. The effluent composition was monitored with the amperometric detector Model 641 (Metrohm, Herisau, Switzerland) with a three-electrode cell ("wall-jet" arrangement), connected in series with the UV-photometric detector output. Separations were performed on a 250 mm × 4 mm I.D. column, packed with Separon SGX $C_{18}$, 7 $\mu$m (Laboratory Instruments, Prague, Czechoslovakia). The mobile phase was 0.1 *M* citrate phosphate buffer (pH 3.1) with varying ethanol content. The flow-rate was 1.0 ml/min. The column was maintained at 25°C.

Detector signals were processed by means of the on-line chromatographic data system Chromatographics 2 (Perkin-Elmer). Calculation of the course of the optimization function, reconstruction of chromatograms, formation of separation maps and other processisng of chromatographic data were performed by means of the COST program written in our laboratory in Basic.

## RESULTS AND DISCUSSION

When studying the metabolism of indole derivatives, it is usually sufficient to analyse a limited number of compounds. When the compounds studied change frequently, it is always necessary to adjust the conditions for optimum separation by means of time-consuming experimental procedures. This approach has been replaced by an optimization method, in which a generally applicable algorithm, based on the use of the COST program, and retention and detection databases for all compounds to be determined are used.

The chromatographic data introduced into the database, retention and column efficiency (expressed as the peak width at one-half the peak height), were obtained for three different ethanol concentrations in the mobile phase. Results for two groups of indole derivatives are presented in Table I. After conversion of the retention times into capacity factors, the dependence on the mobile phase composition was approximated by means of a third-degree polynomial. The peak-width dependence was processed in a similar way. The peak width at one-half the peak height, $w_h$, was calculated from the area, *A*, and height, *H*, of the peak, using the relationship[16].

$$w_h = 2\sqrt{\ln 2/\pi \cdot \frac{A}{H}} \quad (1)$$

TABLE I

RETENTION DATA (CAPACITY FACTORS) FOR THE SUBSTANCES STUDIED

| *Compound* | | *Ethanol (%, v/v)* | | |
|---|---|---|---|---|
| | | *10* | *20* | *30* |
| I | 5-Hydroxytryptophan | 1.004 | 0.429 | 0.215 |
| II | Psilocybin | 1.004 | 0.644 | 0.361 |
| III | 5-Hydroxytryptamine | 1.253 | 0.502 | 0.309 |
| IV | 5-Hydroxy-N,N-dimethyltryptamine | 1.931 | 0.790 | 0.502 |
| V | Tryptophan | 2.721 | 1.146 | 0.644 |
| VI | Tryptamine | 4.043 | 2.004 | 0.931 |
| VII | 5-Hydroxy-N-acetyltryptamine | 4.901 | 1.575 | 0.717 |
| VIII | 5-Methoxytryptamine | 5.223 | 1.717 | 0.790 |
| IX | N-Methyltryptamine | 5.223 | 2.004 | 1.039 |
| X | N,N-Dimethyltryptamine | 6.940 | 2.433 | 1.361 |
| XI | 5-Methoxy-N,N-dimethyltryptamine | 9.013 | 2.648 | 1.146 |
| | | *20* | *30* | *40* |
| XII | 3-Indoleglyoxylic acid | 1.790 | 1.146 | 0.429 |
| XIII | Indole-3-acetamide | 3.292 | 1.575 | 0.717 |
| XIV | Indolelactic acid | 4.545 | 1.897 | 0.790 |
| XV | Indole-3-carbinol | 3.648 | 1.755 | 0.897 |
| XVI | Indole-5-carboxylic acid | 7.442 | 2.755 | 1.073 |
| XVII | 3-Indoleglyoxylamide | 7.155 | 2.936 | 1.146 |
| XVIII | Indole-3-acetic acid | 7.403 | 3.004 | 1.219 |
| XIX | 3-Indoleethanol | 7.369 | 3.077 | 1.288 |
| XX | Indole-3-carboxylic acid | 8.442 | 3.292 | 1.288 |
| XXI | Indole-3-carbaldehyde | 8.356 | 3.541 | 1.433 |
| XXII | Indol-3-acetaldehyde | 8.730 | 3.794 | 1.575 |
| XXIII | 3-Indolylacetonitrile | 11.876 | 4.506 | 1.717 |
| XXIV | Indol-3-acetone | 11.232 | 4.365 | 1.790 |
| XXV | Indole-3-pyruvic acid | 1.824 | 6.511 | 1.790 |
| XXVI | Indole-2-carboxylic acid | 17.670 | 6.155 | 2.146 |
| XXVII | 3-Indolebutyric acid | 29.043 | 9.515 | 2.863 |
| XXVIII | 3-Indolyl acetate | 3.433 | 7.940 | 2.936 |

For each compound, the parameters of the regression polynomials were stored in the computer memory.

The detection data are represented by values of the half-wave potentials, determined by evaluating hydrodynamic voltammograms. Whereas the UV-photometric detector responds to all compounds eluted from the column, the electrochemical detector responds only to a limited number of compounds. The selectivity can be altered by chosing a suitable working potential of the electrode system. Depending on the shape of the polarization curve, the potential used for the detection of a given compound should be by 0.2–0.3 V higher than the half-wave potential. The course of hydrodynamic voltammograms for typical indole derivatives is shown in Fig. 1.

The optimization procedure consists of five steps: (1) selection of compounds to be optimized; (2) display of the relationship between the capacity factors and the content of the organic modifier in the mobile phase; (3) representation of the course of the chromatographic optimization function (COF) and determination of the op-

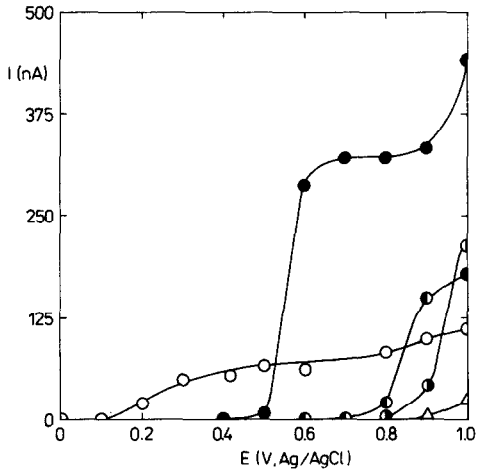


Fig. 1. Typical hydrodynamic voltammograms. Compounds: indol-3-acetone (○); 5-hydroxytryptophan (●); indolelactic acid (◐); tryptamine (◑) and indole-5-carboxylic acid (△). For experimental conditions see the text.

timum; (4) occasional simulation of the chromatogram for the proposed mobile phase composition; (5) construction of separation maps.

The chromatographic optimization function (COF)[17] includes parameters for the total separation and time requirements of the analysis

$$\mathrm{COF} = \sum_{i=1}^{N-1} \ln \frac{R_i}{R_c} + v\,|T_M - T_L| \qquad (2)$$

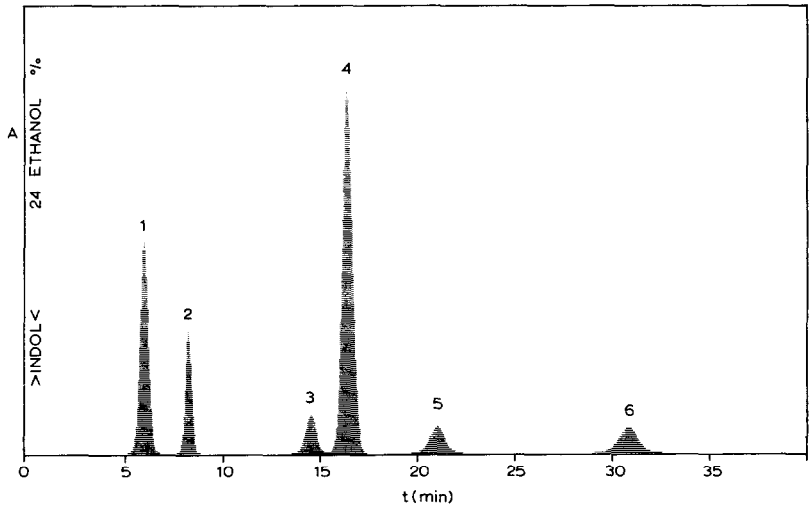


Fig. 2. Simulated chromatogram generated by the COST program. Peaks: 1 = 3-indoleglyoxylic acid; 2 = indol-3-acetamide; 3 = indole-5-carboxylic acid; 4 = indole-3-carboxylic acid; 5 = indol-3-acetone; 6 = indole-2-carboxylic acid. Reconstruction for 24% (v/v) ethanol in the mobile phase.

where $N$ is the number of compounds in the group to be optimized, $R_i$ is resolution of the $i$th and $(i+1)$th peak (of peaks $i$ and $i+1$), $R_c$ is the required resolution, $v$ is a weighing factor, $T_M$ is the maximum acceptable analysis time and $T_L$ is the retention time of the last compound eluted. The critical resolution was chosen as 1.5, the time weighing factor as 0.1 and the maximum acceptable analysis time was usually 20 min. The optimum composition of the mobile phase is determined by the position of the maximum in the chromatographic optimization function.

For visual control of a separation under the experimental conditions proposed, hypothetical chromatograms were simulated. In the graphic interpretation the relative retention times and peaks widths at one-half the height of individual peaks were preserved. However, on reconstruction, the real shape was substituted by the ideal Gaussian curve described by

$$Y = H_{max} \cdot \exp\{-0.5[(T-T_R) \cdot 2.3548/w_h]^2\} \quad (3)$$

where $H_{max}$ is the peak height, $T$ the time, $T_R$ the retention time and $w_h$ is the half-width of the peak (width for $Y = 1/2\ H_{max}$). An example of the simulated chromatogram for a model mixture of six indole derivatives is shown in Fig. 2. The real and simulated chromatograms are always slightly different. However, the above simulation is adequate for evaluating the conditions proposed.

Separation tables were prepared for rapid evaluation of the resolution of various compounds. A chromatographic separation table can be constructed for any modifier content in the mobile phase. A selectable resolution is the criterion used for deciding on the solvent composition. In the separation table, airs of compounds fulfilling the criterion of sufficient resolution are differentiated graphically. The voltammetric separation table is constructed in a similar way. However, the difference in half-wave potentials of individual compounds served as the criterion. A difference of about 0.3 V is usually sufficient for the selective detection (non-chromatographic

TABLE II

TOTAL SEPARATION TABLE FOR FIFTEEN SUBSTANCES OF INTEREST

| *Compound* | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* | *10* | *11* | *12* | *13* | *14* | *15* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 Indole-3-pyruvic acid | X | | | | | | | | | | | | | | |
| 2 Indol-3-acetamide | | X | | | | | | | | | | | | | |
| 3 Indolelactic acid | | | X | | | | | | | | | | | | |
| 4 Indolyl acetate | | | | X | | X | | | | | X | | | | |
| 5 3-Indoleglyoxylamide | | | | | X | | X | X | | | | | | | |
| 6 3-Indoleethanol | | | | X | | X | | | | | X | | | | |
| 7 Indol-3-acetic acid | | | | | X | | X | X | X | | | | | | |
| 8 Indole-5-carboxylic acid | | | | | X | | X | X | X | | | | | | |
| 9 Indole-3-carbaldehyde | | | | | | | | | X | | | | | | |
| 10 Indole-3-carboxylic acid | | | | | | | | | | X | X | | | | |
| 11 Indol-3-acetaldehyde | | | | | | | | | | X | X | | | | |
| 12 Indol-3-acetone | | | | | | | | | | | | X | | | |
| 13 3-Indolylacetonitrile | | | | | | | | | | | | | X | | |
| 14 Indole-2-carboxylic acid | | | | | | | | | | | | | | X | |
| 15 3-Indolebutyric acid | | | | | | | | | | | | | | | X |

separation) of indole derivatives. Voltammograms for all the compounds of interest must be recorded and evaluated in order to construct the non-chromatographic separation table.

"The total separation table" (Table II) represents the separability of pairs of compounds by means of the combined chromatographic and non-chromatographic method, implemented by selective detection. The "total table" utilizes the two separation tables described here, being its logical sum. The character X designates pairs of compounds that cannot be separated chromatographically under the given conditions (the critical resolution was set at 1.5) or by selective detection (difference in potentials for detection of 0.3 V).

The analysis was evaluated according to whether the compounds to be determined can be separated on a column or whether selective detection must be used. In the former case, the evaluation was performed in the usual way. The ratio of the

TABLE III

DETECTION DATA FOR THE SUBSTANCES

For compound identifications see Table I.

| *Compound* | *Detection limit (ng)* | | UV/ED*** | $E_{\frac{1}{2}}$§,§§ *(V)* | *E(det)*§ *(V)* |
|---|---|---|---|---|---|
| | *UV** | *ED*** | | | |
| I | 20 | 0.5 | 39.2 | 0.55 | 0.7 |
| II | 20 | 1.0 | 20.6 | 0.90 | 1.0 |
| III | 64 | 2.6 | 24.6 | 0.60 | 0.7 |
| IV | 34 | 1.0 | 34.0 | 0.55 | 0.7 |
| V | 88 | 2.8 | 31.4 | 0.95 | 1.0 |
| VI | 30 | 1.0 | 30.0 | 0.95 | 1.0 |
| VII | 27 | 0.7 | 36.5 | 0.55 | 0.7 |
| VIII | 32 | 0.9 | 34.4 | 0.95 | 1.0 |
| IX | 44 | 1.5 | 29.3 | 0.95 | 1.0 |
| X | 22 | 0.7 | 31.4 | 1.00 | 1.0 |
| XIII | 27 | 1.0 | 27.0 | 0.95 | 1.0 |
| XIV | 46 | 1.2 | 38.3 | 0.85 | 1.0 |
| XVI | 52 | – | – | – | – |
| XVII | 33 | – | – | – | – |
| XVIII | 32 | – | – | – | – |
| XIX | 47 | 0.8 | 61.8 | 0.90 | 1.0 |
| XX | 49 | 1.3 | 37.7 | 0.85 | 1.0 |
| XXI | 29 | – | – | – | – |
| XXII | 96 | 2.4 | 40.0 | 0.85 | 1.0 |
| XXIII | 130 | – | – | – | – |
| XXIV | 57 | 3.2 | 17.8 | 0.20 | 0.4 |
| XXV | 22 | 2.3 | 9.6 | 0.90 | 1.0 |
| XXVI | 35 | 1.1 | 31.8 | 0.80 | 1.0 |
| XXVII | 220 | 4.8 | 45.8 | 0.85 | 1.0 |
| XXVIII | 90 | 2.2 | 40.9 | 0.85 | 1.0 |

* Detection limit of UV-photometric detector at 267 nm for a signal-to-noise ratio of 2.
** Detection limit of voltammetric detector at the potential *E*(det).
*** Detection limit ratio.
§ Potential measured *versus* the saturated calomel electrode.
§§ Half-wave potential from the hydrodynamic voltammogram.

signals of the photometric and of the electrochemical detector can be used to control the purity (homogeneity) of the peak. Compared with measurements at two wavelengths, the procedure employed is less reliable, due to the lower stability of the electrochemical detector signal. When the compounds could not be separated chromatographically but were selectively detectable, the records of the signals from both detectors were gradually evaluated. When it was impossible to resolve two compounds, even by selective detection, the representation of the compounds in the peak was determined by calculation. In a calibration experiment, it was first necessary to determine the response factors of both compounds in both detectors used. As the errors in a series of measurements are reflected in a single determination, this greatly diminished the reliability of the results.

The possibility of using the experimental arrangement described for quantitative analysis was also investigated. Detection limits for a signal-to-noise ratio of 2 were determined, both for the UV-photometric (at 267 nm) and the electrochemical (at the detection potential) detector. The results are summarized in Table III. Whereas the detection limits for the UV-photometric detector varied within a range of several nanograms, even tenths of nanograms of certain compounds can be determined with the electrochemical detector. With the exception of five compounds that do not respond to electrochemical detection, the sensitivity of the electrochemical detector is about 1.5 orders of magnitude higher than that of the UV detector for all compounds studied. For a comparison of the two detectors, the ratio of the corresponding detection limits is presented in Table III.

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