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# New methods for spectrometric peak purity analysis in chromatography<sup>1</sup>

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## Abstract

Simple methods for peak purity analysis in chromatography are proposed for accurately assaying the chromatographic or electrophoretic process by spectroscopy. With these methods, the purity at each point of a chromatographic peak containing an unknown type and number of components can be detected and visualized. The 'absorbance (A) diagram' is constructed by plotting the absorbance values of different wavelengths against each other. When only straight lines are obtained for different wavelength combinations, only one component was detected in the corresponding peak. Bent curves in the A diagrams mean that at least two components were registered. Straight lines in the absorbance difference quotient (ADQ) diagrams indicate that two components occur in the corresponding peak. Bent curves signify that at least three components were registered. The new methods are demonstrated using the HPLC technique for the separation of chlorogenic acid and epicatechin. Integral A and ADQ diagrams and the corresponding mean diagrams complete the new concept. © 1998 Elsevier Science B.V.

**Keywords:** Peak purity; Absorbance diagram; Absorbance difference quotient diagram; Chlorogenic acid; Epicatechin

## 1. Introduction

The basic question of each chromatographic separation is: How many components were detected in each chromatographic peak? If a peak does not only contain the component that is of special interest (perhaps it contains others), then the separation method should be improved.

Because of the importance of peak purity, several powerful tools have been developed. A series of numerical methods have been introduced, such as [1–5]:

- Ratioing
- Spectral discriminators
- MARC parameters
- Peak purity index
- Peak deconvolution
- Multicomponent analysis
- Comparison of spectra by chemometrics.

Most of these methods are of high quality and are sophisticatedly developed. However, most of them are strictly numerical which leads to some disadvantages:

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<sup>1</sup> Dedicated to Prof. Dr. E. Bayer on the occasion of his 70th birthday.

- They must be integrated in devices as ‘black-box unities’ and the user can apply these methods only in a schematic routine without any modifications.
- In the case of contradictory reports of different methods, the user cannot decide which result has importance.
- Numerical artefacts and problems which are mathematically poorly conditioned cannot easily be recognised in routine applications.

There is an urgent demand for a simple but nevertheless efficient method which leads to a graphic analysis of peak purity. The method should be easily comprehensible for the user and should demand only a little basic knowledge.

The basic question of chromatography concerning the number of components detected in a peak can be focussed here in the question: Which information can be obtained solely by spectroscopic data (i.e. the absorbance data registered)?

This question generally means that no spectroscopic information is known about any individual component. This standpoint may be helpful, for example, when an unknown substance is to be isolated from a biological liquid and which is then to be analyzed later.

The absorbance (A), absorbance difference (AD) and absorbance difference quotient (ADQ) diagrams are efficient tools for the evaluation of spectrometric data. These diagrams were advantageously applied to the analysis of reaction systems [6,7] and titration equilibria [8]. Although these diagrams can be used whenever the spectrometric data obey the generalized law according to Lambert–Beer–Bouguer [6–8] they were not applied to spectroscopically recorded chromatograms until now. The construction of A, AD and ADQ diagrams is very simple. The efficiency of these plots for the analysis of spectroscopically registered chromatographic peaks is demonstrated subsequently. The knowledge about spectra or molar absorptivities of individual components is not necessary for the application of the presented methods. With additional informations about some molar absorptivities or concentrations of two mixed samples the concentration quotient of the mixture during the chromatographic separation process can be detected [9].

## 2. Theory

### 2.1. The basic equation

The generalized law according to Lambert–Beer–Bouguer holds up [6–8]:

$$A_{\lambda} = l \cdot \sum_{i=1}^s \epsilon_{\lambda i} \cdot c_i \quad (1)$$

( $A_{\lambda}$  = absorbance at a particular wavelength  $\lambda$ ,  $l$  = path-length of cell,  $\epsilon_{\lambda i}$  = molar absorptivity of the absorbing species  $i$ ,  $c_i$  = concentration of species  $i$  at wavelength  $\lambda$ ,  $s$  = maximum number of absorbing components separated).

During the chromatographic process  $A_{\lambda}$  changes in dependence on retention time  $t$ . Each chromatogram has its individual baseline. This line can be stable or can drift during the time. Taking the baseline into account the general equation for chromatography here is:

$$A_{\lambda}(t) = l \cdot \sum_{i=1}^s \epsilon_{\lambda i} \cdot c_i(t) + A_{\lambda}^B(t) \quad (2)$$

$A_{\lambda}^B(t)$  is the absorbance of the baseline (Index B) at wavelength  $\lambda$ . Three possibilities exist for the value of  $A_{\lambda}^B(t)$ :

$$A_{\lambda}^B(t) = 0,$$

$$A_{\lambda}^B(t) = \text{const. and},$$

$$A_{\lambda}^B(t) \neq \text{const. .}$$

All these cases are realized in chromatography. Therefore, they have to be taken into consideration for a general evaluation method.

According to Eq. (2) the absorbance difference  $\Delta A_{\lambda}$  can be introduced as follows:

$$\Delta A_{\lambda} = A_{\lambda}(t) - A_{\lambda}^B(t) = l \cdot \sum_{i=1}^s \epsilon_{\lambda i} \cdot c_i(t) \quad (3)$$

Here,  $\Delta A_{\lambda}$  corresponds to the individual absorbing species  $i$  in the same manner as  $A_{\lambda}$  does according to Eq. (1). Eq. (3) is the basic equation for the introduction of A, AD and ADQ diagrams and corresponding plots in chromatography.

## 2.2. A and AD diagrams

For the following consideration it is assumed that a separation between the different components of the system took place during the chromatographic process at least to some extent. In the special case of the separation of the particular component being completely carried out, only one single separated component is recorded by the optical detector. Each point of the corresponding peak can be described by the following terms according to Eq. (3) ( $s=1$ ):

$$\Delta A_\lambda = A_\lambda(t) - A_\lambda^B(t) = l \cdot \epsilon_{\lambda 1} \cdot c_1(t) \quad (4)$$

This relationship is true for all wavelengths of the spectrum which were recorded at time  $t$ . When two wavelengths ( $\lambda_1, \lambda_2$ ) with a relatively high absorbance are selected, it follows from Eq. (4) for the corresponding measured values that:

$$\Delta A_1 := \Delta A_{\lambda_1} = l \cdot \epsilon_{\lambda_1 1} \cdot c_1 = l \cdot \epsilon_{11} \cdot c_1$$

and

$$\Delta A_2 := \Delta A_{\lambda_2} = l \cdot \epsilon_{\lambda_2 1} \cdot c_1 = l \cdot \epsilon_{21} \cdot c_1$$

By division of these two equations, one obtains:

$$\frac{\Delta A_1}{\Delta A_2} = \frac{A_1(t) - A_1^B(t)}{A_2(t) - A_2^B(t)} = \frac{\epsilon_{11}}{\epsilon_{21}} \quad (5)$$

It follows from this:

$$\Delta A_1 = \frac{\epsilon_{11}}{\epsilon_{21}} \cdot \Delta A_2 \quad (6)$$

In the absorbance difference (AD) diagram the quantities  $\Delta A_1$  and  $\Delta A_2$  belonging to different retention times are plotted against each other. All points must lie on a straight line according to Eq. (6). The points start from the origin of the coordinate system and pass on a straight line during the evolution of the peak. When the peak maximum is reached the points begin to return on the same line to the origin.

The rearrangement of Eq. (6) leads to:

$$A_1(t) = \frac{\epsilon_{11}}{\epsilon_{21}} \cdot A_2(t) - \frac{\epsilon_{11}}{\epsilon_{21}} \cdot A_2^B(t) + A_1^B(t) \quad (7)$$

In the case of absorbance (A) diagram the plot  $A_1(t)$  vs.  $A_2(t)$  is constructed. When  $A_\lambda^B$  (with  $\lambda=1$ ,

2) is constant a straight line is obtained with the same slope as the corresponding line in the AD diagram. If  $A_2^{BB}=0$ , a line passing through the origin results according to:

$$A_1(t) = \frac{\epsilon_{11}}{\epsilon_{21}} \cdot A_2(t).$$

These results show that peak purity can be tested very simply by A and AD diagrams.

## 2.3. ADQ diagrams

If two components have not completely been separated by chromatography the absorbance can be described by the following equation according to Eq. (3) ( $s=2$ ):

$$\Delta A_\lambda = A_\lambda(t) - A_\lambda^B(t) = l \cdot (\epsilon_{\lambda 1} c_1 + \epsilon_{\lambda 2} \cdot c_2) \quad (8)$$

In order to get a linear relation between the measured values, Eq. (8) is applied for three wavelengths ( $\lambda=1, 2, 3$ ):

$$\Delta A_1 = l \cdot (\epsilon_{11} \cdot c_1 + \epsilon_{12} \cdot c_2)$$

$$\Delta A_2 = l \cdot (\epsilon_{21} \cdot c_1 + \epsilon_{22} \cdot c_2)$$

$$\Delta A_3 = l \cdot (\epsilon_{31} \cdot c_1 + \epsilon_{32} \cdot c_2).$$

From these three equations, the concentrations  $c_1$  and  $c_2$  can be eliminated leading to the equation:

$$\Delta A_1 = \alpha_1 \cdot \Delta A_2 + \alpha_2 \cdot \Delta A_3 \quad (9)$$

with

$$\alpha_1 = \frac{\epsilon_{11} \epsilon_{32} - \epsilon_{12} \epsilon_{31}}{\epsilon_{21} \epsilon_{32} - \epsilon_{22} \epsilon_{31}} \text{ and } \alpha_2 = - \frac{\epsilon_{11} \epsilon_{22} - \epsilon_{12} \epsilon_{21}}{\epsilon_{21} \epsilon_{32} - \epsilon_{22} \epsilon_{31}}$$

It follows from this equation that:

$$\frac{\Delta A_1}{\Delta A_2} = \alpha_1 + \alpha_2 \cdot \frac{\Delta A_3}{\Delta A_2} \quad (10)$$

The absorbance difference quotient (ADQ) diagram is the plot of  $(\Delta A_1)/(\Delta A_2)$  vs.  $(\Delta A_3)/(\Delta A_2)$ . A straight line results for the case  $s=2$ . The presence

of two components in chromatographic peaks can thus be detected in a graphic procedure. A bent curve is obtained if  $s > 2$ .

In analogy to the ADQ diagram, the so-called ADQ3, ADQ4, ..., ADQ $s$  diagrams [8] can be constructed for the identification of 3, 4, ...,  $s$  components in chromatographic peaks.

If  $A_\lambda^B$  is zero at all wavelengths, Eq. (10) is reduced to:

$$\frac{A_1}{A_2} = \alpha_1 + \alpha_2 \cdot \frac{A_3}{A_2} \quad (11)$$

The plot  $(A_1)/(A_2)$  vs.  $(A_3)/(A_2)$  is called the 'absorbance quotient (AQ) diagram'.

#### 2.4. Integral A, AD, AQ and ADQ diagrams

Diode array spectrometers are favoured for use in spectrometric chromatography since they can register a spectrum in the range of milliseconds. However, they have less sensitivity compared with conventional UV-Vis spectrometers. This disadvantage can at least partially be compensated by the construction of 'integral diagrams' introduced here in analogy to the A, AD, AQ and ADQ diagrams.

Fluctuation of measured values caused by a nonoptimal signal-to-noise ratio can be improved by smoothing the corresponding spectrum ( $A$  vs.  $\lambda$ ) in a selected wavelength range ( $\lambda_j - \lambda_i$ ) using polynomial functions, for example. Thus, it is possible to determine areas under the curve  $A$  vs.  $\lambda$  which are established by different segments on the wavelength axis. These areas can be evaluated in analogy to the individual  $A_\lambda$  values as demonstrated in Sections 2.2 and 2.3.

Considering the case  $s = 1$  under this aspect the integration of Eq. (4) leads to the expression:

$$\int_{\lambda_i}^{\lambda_j} \Delta A_\lambda d\lambda = l \cdot c_1(t) \cdot \int_{\lambda_i}^{\lambda_j} \epsilon_{\lambda 1} d\lambda \quad (12)$$

The evaluation of two segments of the wavelength axis ( $\lambda_2 - \lambda_1$  and  $\lambda_4 - \lambda_3$ ) can be analogously carried out as demonstrated in Eq. (6):

$$\begin{aligned} \int_{\lambda_1}^{\lambda_2} \Delta A_\lambda d\lambda &= \left[ \frac{\int_{\lambda_1}^{\lambda_2} \epsilon_{\lambda 1} d\lambda}{\int_{\lambda_3}^{\lambda_4} \epsilon_{\lambda 1} d\lambda} \right] \cdot \int_{\lambda_3}^{\lambda_4} \Delta A_\lambda d\lambda \\ &= \beta \cdot \int_{\lambda_3}^{\lambda_4} \Delta A_\lambda d\lambda \quad (13) \end{aligned}$$

By plotting the integral quantities against each other in a diagram, the so called 'integral absorbance difference (iAD) diagram' is produced. In the case of  $s = 1$ , a straight line is obtained passing through the origin with slope  $\beta$ .

If  $A_\lambda^B$  is constant in the considered wavelength ranges this quantity can be separated in Eq. (13) leading to the expression:

$$\begin{aligned} \int_{\lambda_1}^{\lambda_2} A_\lambda d\lambda &= \beta \cdot \int_{\lambda_3}^{\lambda_4} A_\lambda d\lambda + A_{\lambda_2}^B (\lambda_2 - \lambda_1) - \beta \cdot A_{\lambda_4}^B \\ &\quad \cdot (\lambda_4 - \lambda_3) \quad (14) \end{aligned}$$

The plot  $\int_{\lambda_1}^{\lambda_2} A_\lambda d\lambda$  vs.  $\int_{\lambda_3}^{\lambda_4} A_\lambda d\lambda$  is called the 'integral absorbance (iA) diagram'.

In analogy to the ADQ diagrams the 'integral absorbance difference quotient (iADQ) diagrams' can be constructed for the case  $s = 2$  [compare Eq. (10)].

$$\frac{\int_{\lambda_3}^{\lambda_4} \Delta A_\lambda d\lambda}{\int_{\lambda_2}^{\lambda_3} \Delta A_\lambda d\lambda} = \beta_1 + \beta_2 \cdot \frac{\int_{\lambda_5}^{\lambda_6} \Delta A_\lambda d\lambda}{\int_{\lambda_1}^{\lambda_2} \Delta A_\lambda d\lambda} \quad (15)$$

( $\beta_1$  and  $\beta_2$  are constants).

The corresponding 'integral absorbance quotient (iAQ) diagrams' are obtained for the case  $A_\lambda^B = 0$ .

#### 2.5. Mean A, AD, AQ and ADQ diagrams

The well known averaging method which is

applied to absorbance values can also be transferred to the concept developed. The procedure is subsequently described using the case  $s=2$ . The application of Eq. (8) for  $n$  wavelengths leads to:

$$\begin{aligned} \Delta A_1 &= l(\epsilon_{11}c_1 + \epsilon_{12}c_2) \\ &\vdots \\ \Delta A_n &= l(\epsilon_{n1}c_1 + \epsilon_{n2}c_2) \end{aligned} \quad (16)$$

The averaging method applied to the  $n$  values  $\Delta A_i$  supplies the mean value  $\overline{\Delta A_1}$  which is defined by:

$$\begin{aligned} \overline{\Delta A_1} &= \frac{1}{n} \sum_{i=1}^n \Delta A_i = \frac{l}{n} \left[ c_1 \sum_{i=1}^n \epsilon_{i1} + c_2 \sum_{i=1}^n \epsilon_{i2} \right] \\ &= \sigma_{11}c_1 + \sigma_{12}c_2 \end{aligned} \quad (17)$$

It is assumed that the  $n$  wavelengths are arranged behind each other and represent a characteristic segment of the spectrum  $A$  vs.  $\lambda$  at time  $t$ .

Eq. (17) can be developed for three wavelength segments according to:

$$\begin{aligned} \overline{\Delta A_1} &= \sigma_{11}c_1 + \sigma_{12}c_2 \\ \overline{\Delta A_2} &= \sigma_{21}c_1 + \sigma_{22}c_2 \\ \overline{\Delta A_3} &= \sigma_{31}c_1 + \sigma_{32}c_2 \end{aligned} \quad (18)$$

The wavelength range ( $\Delta\lambda$ ) for all three segments can be different. In analogy to Eq. (10) one obtains from Eq. (18):

$$\frac{\overline{\Delta A_1}}{\overline{\Delta A_2}} = \gamma_1 + \gamma_2 \frac{\overline{\Delta A_3}}{\overline{\Delta A_2}} \quad (19)$$

$\gamma_1$  and  $\gamma_2$  are constants [compare  $\alpha_1$  and  $\alpha_2$  in Eq. (9)] which depend only on  $\sigma_{ij}$ .

The plot  $\frac{\overline{\Delta A_1}}{\overline{\Delta A_2}}$  vs.  $\frac{\overline{\Delta A_3}}{\overline{\Delta A_2}}$  is called the ‘mean ADQ (mADQ) diagram’. The corresponding ‘mean AQ (mAQ) diagram’ is obtained when  $A_\lambda^B$  is zero for all used wavelengths.

In analogy to mAQ and mADQ diagrams, the corresponding plots mA and mAD can be constructed.

The applications of Eqs. (19), (9), (15) lead to the same result in peak analysis. When only straight lines

are obtained, the case  $s=2$  is true. The Eqs. (19), (9) and (15) differ in their functions. In Eq. (15) the wavelengths are directly involved in the corresponding terms meanwhile the wavelengths occur only in an indirect way within Eqs. (19) and (9).

The propagation of errors concerning the evaluation of absorbance data depends on the function which is applied. Thus, the three developed methods (see Sections 2.3, 2.4 and 2.5) represent basically different limits in their efficiency.

### 3. Experimental

#### 3.1. Chemicals and instruments

Spectrometric chromatographic investigations were carried out with chlorogenic acid and epicatechin. Chlorogenic acid was purchased by ROTH (Karlsruhe, Germany). Epicatechin was extracted from horse chestnuts at the Lehrstuhl für Obstbau (TU-München). The chromatographic separation was carried out with a HPLC installation from GYN-KOTEK (Germersheim, Germany) using a high-precision pump (model 300 C) and a gradient system (250 B) of low pressure. An auto sampler (model 231) from Gilson–Abimed (Langenfeld) was employed. The optical signals were registered by a photodiode array detector (model 1040 A) from Hewlett–Packard (Waldbrunn) using the evaluation software HP 9000. A reversed-phase column (Hypersil ODS C<sub>18</sub>, 250×4.6 mm) was applied (particle size, 3 μm; flow-rate 1 ml/min). An isocratic gradient was established consisting of 40% methanol and 60% distilled water (water with 1% acetic acid). The samples were dissolved in methanol. The concentration of chlorogenic acid amounted to 1.3 mg/ml, the epicatechin concentration was 2 mg/ml. Ten microlitres of the methanolic solution were used for each HPLC investigation. The mixture contained 5 μl of the chlorogenic acid solution and 5 μl epicatechin solution.

#### 3.2. Peaks with one absorbing component ( $s=1$ )

A pure substrate was injected into a HPLC reversed-phase column for the simulation of a com-

pletely chromatographic separation. At first, chlorogenic acid was employed.

The spectrometric evaluation of the chromatographic peak obtained, according to the concept of A diagrams is shown in Fig. 1. The baselines were constant ( $A_\lambda^B = \text{const.} = 1.5 \text{ mAU}$ ) for all wavelengths and had a value near zero. Three straight lines are presented in Fig. 1 for four wavelengths. The points scatter only slightly about the straight lines drawn in Fig. 1 and calculated by linear regression. The points start near the origin and move along these straight lines in the same measure as the three dimensional chromatographic peak ( $A$  vs.  $\lambda$  vs.  $t$ ) is forming. The longest distance to the origin is obtained when the peak maximum is reached. The points return on the same straight line when the peak begins to disappear. The shape of the peak has no influence on the position of the straight lines i.e. the peak ( $A_\lambda$  vs.  $t$ ) may have the shape of a Gaussian, Lorentz or Poisson curve. The A diagram is independent of the extent of peak tailing.

The investigation of epicatechin instead of chloro-

genic acid leads to similar results as shown by Fig. 1 for chlorogenic acid.

The construction of A diagrams is presumably the simplest method of peak purity analysis. However, it is necessary to construct several A diagrams in order to be sure that a foreign substrate would have been detected in the examined wavelength range if it had existed. Therefore, it is recommended to select 4–10 wavelengths for the construction of A diagrams. The change of absorbance should be sufficiently high at these wavelengths and the wavelength should be spread over the whole spectra registered.

If only straight lines are obtained in the constructed A diagrams only one component was spectroscopically registered. In that case, the chromatographic separation was complete according to the detection system. The significance of A diagrams is very high. The slightest deviations from a linear run being systematic can be precisely recognized in a visual manner. No great experience is necessary for the construction of A diagrams.

The A diagrams are preferably constructed when

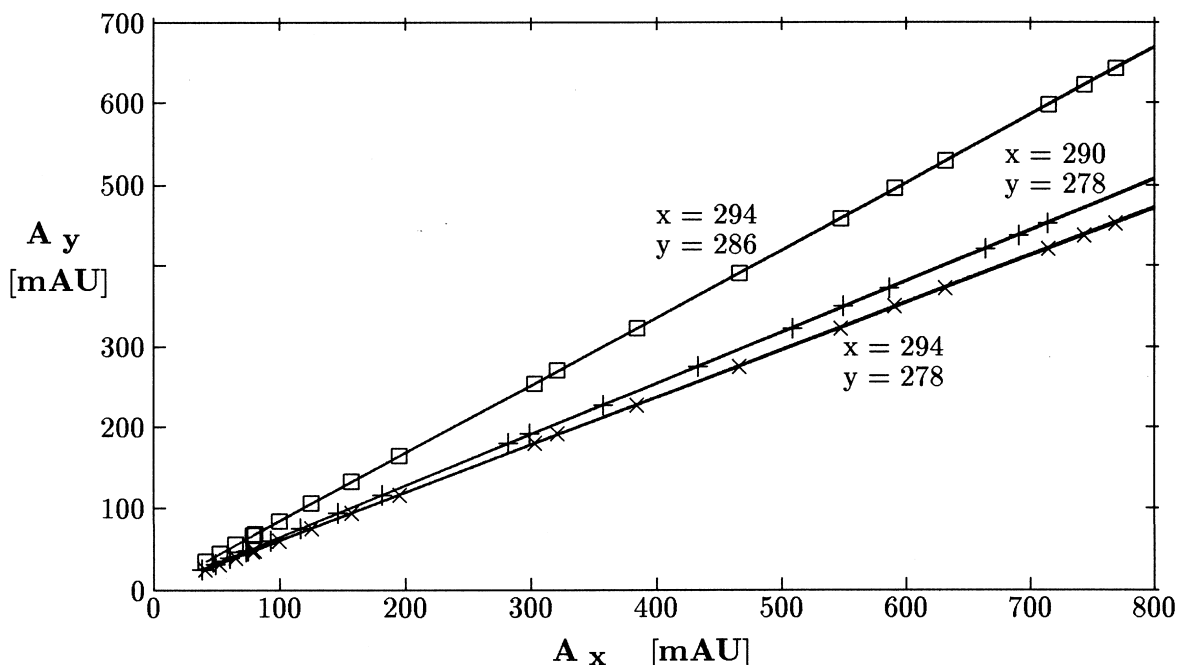


Fig. 1. Diagram of chlorogenic acid. The letters  $x$  and  $y$  declare the wavelengths (unit nm), the absorbances of which were used for the construction of the corresponding A diagram. mAU = milli absorbance unit. Values lying very closely to the origin were omitted.

spectroscopic measurement data are to be directly evaluated without any data preprocessing. Each data manipulation can principally lead to systematic errors. This danger is excluded here by the construction of A diagrams. In the case of baseline correction, the original data are changed (even if to a small extent in most cases). Thus, the evaluation of these manipulated data does not lead to the A diagrams.

The A and AD diagrams form the basis for the evaluation of spectroscopic measurement data. The well known method of ratioing is a part of this concept. According to the method of ratioing, strict peak homogeneity ( $s=1$ ) exists when the ratio  $[A_1(t)]/[A_2(t)]$  remains constant during the chromatographic process. This ratio is identical with the slope of that straight line which is obtained by the corresponding A diagram  $[A_1(t)$  vs.  $A_2(t)]$ . However, this correlation is only true if the straight line passes through the origin of the A diagram. When the baseline at the corresponding wavelengths differ from the value zero ( $A_\lambda^B = \text{const.}$ ) a parallel to this line is the consequence. Then, the method of ratioing leads to wrong results since the ratio  $(A_1)/(A_2)$  changes continuously in dependence on time although the case  $s=1$  exists. These considerations clearly show that in the theory of ratioing, the ratio  $(A_1)/(A_2)$  should be generally replaced by the quotient  $(\Delta A_1)/(\Delta A_2)$ .

The criterion for  $s=1$  concerning the method of ratioing is to get a constant slope for the whole process. In the case of A and AD diagrams, the corresponding criterion is to get a straight line. This distinction is of importance, for example, when the slope is high. Small fluctuations in the absorbance influenced by noise may lead to different values of the slope meanwhile the corresponding points scatter only slightly around a straight line in the A or AD diagram. As well, near to the edges of the chromatographic peak, the slope tends towards 0/0 leading to great errors. The A and AD diagrams are independent of this poorness to a large degree.

In the case of peak homogeneity ( $s=1$ ), the slopes of the straight lines obtained by the corresponding A and AD diagrams are only identical when  $A_\lambda^B$  is constant during the whole chromatographic process. However, when  $A_\lambda^B$  is changing with time, the habitus of the corresponding curves is different in the A and AD diagrams. Then, the A diagrams lead to

bent curves, meanwhile straight lines are observed in the corresponding AD diagrams when  $A_\lambda^B$  was correctly determined with dependence on time. For example,  $A_\lambda^B$  values can be calculated by fitting the corresponding baseline with polynomial functions.

The case  $s=1$  has particular importance for chromatography. In this case, a substance is completely separated from a mixture. The corresponding peak may later be subjected to further investigations, for example, for structure identification. However, the rare case may occur that a peak was homogenous but nevertheless impure. When the impurity has nearly exactly the same physicochemical properties as the substance which is of interest it can be possible that both substances are identically separated. The concentration ratio of both substrates  $[(c_2)/(c_1) = \kappa = \text{const.}]$  remains nearly constant during the whole chromatographic process. Then, the absorbance difference generally obeys the equation:

$$\Delta A_\lambda = l \cdot (\epsilon_{\lambda 1} \cdot c_1 + \epsilon_{\lambda 2} \cdot c_2) = l \cdot c_1 \cdot (\epsilon_{\lambda 1} + \epsilon_{\lambda 2} \cdot \kappa)$$

The corresponding AD diagram leads to a straight line with the slope:

$$\frac{\Delta A_1}{\Delta A_2} = \frac{\epsilon_{11} + \epsilon_{12} \cdot \kappa}{\epsilon_{21} + \epsilon_{22} \cdot \kappa} = \text{const.}$$

Thus, spectroscopic uniformity is presented here.

Different methods for peak homogeneity determination have been developed [10–12]. For example, absorbance equations have been created to nullify signals in order to detect coincident impurities [10]. Absorbance differences which are related to different wavelengths have been introduced for the analysis of poorly separated samples [11]. Graphic techniques and computer-aided strategies for spectral data interpretation have been discussed [12,13]. Finally, procedures for optimizing detector bandwidth, for selecting wavelengths and for applying multiple purity parameters have been described [14,15]. Most of these methods include the case  $s=2$  which is the subject of Section 3.3.

### 3.3. Peaks with two absorbing components ( $s=2$ )

In order to study an incomplete chromatographic separation, a mixture consisting of chlorogenic acid and epicatechin was tested under nonoptimal HPLC

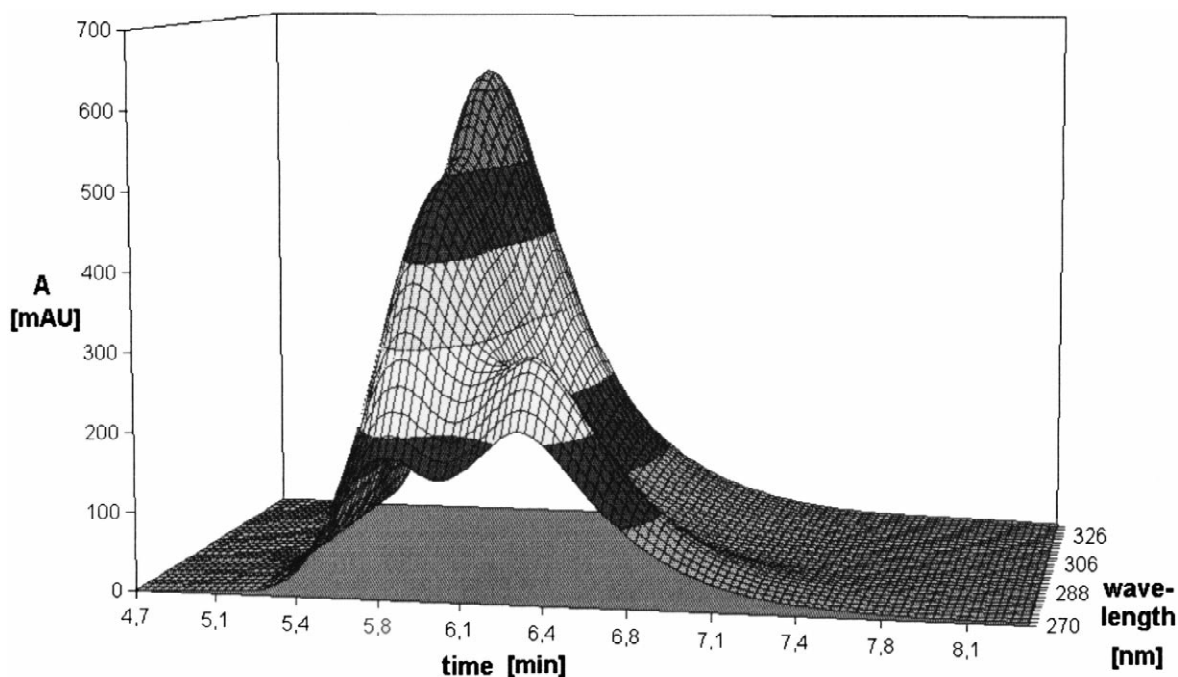


Fig. 2. Diagram A vs.  $\lambda$  vs.  $t$  for a mixture of chlorogenic acid and epicatechin (HPLC).

conditions. In Fig. 2 the result is shown in a three-dimensional plot. As one can immediately recognize, the separation was incomplete. Obviously, the baselines for all wavelengths were constant and the relation  $A_{\lambda}^B \approx 0$  is approximately true ( $A_{\lambda}^B \approx 1.5$  mAU in comparison to  $A_{\max} = 625.6$  mAU).

A typical AD diagram obtained from Fig. 2 is shown in Fig. 3. The absorbance difference  $\Delta A_{\lambda}$  is defined by Eq. (3):

$$\Delta A_{\lambda} = A_{\lambda}(t) - A_{\lambda}^B(t)$$

As demonstrated in Fig. 3, the points start from the origin and move firstly on a straight line (see arrow pointing to the right side). After reaching a maximum distance to the origin, some points return in direction to the origin on the same straight line (see arrow orientated to the left side near the top of the straight line drawn in Fig. 3). After a short distance the points move away from the straight line. Passing over a maximum, the points then return to the origin. The last points lie on a straight line again.

Because of the nonlinear curve in Fig. 3, at least

two components were spectroscopically registered inside the chromatographic peak ( $s \geq 2$ ). The proof, that  $s=2$  is true, is given by the ADQ diagrams according to Eq. (10). As shown in Fig. 4 strictly linear curves are obtained for different wavelength combinations. As in the case of A diagrams, it is necessary to construct about 4–10 curves for the ADQ diagram as well.

Straight lines in the ADQ diagram generally indicate that two components occur in the corresponding peak. However, two exceptions must be taken into consideration [6–8]:

- Straight lines passing through the origin of ADQ diagrams are degenerated and do not indicate the case  $s=2$  (compare Eq. (10): if  $\alpha_1=0$  then  $\Delta A_1 = \alpha_2 \cdot \Delta A_3$  is true; for example,  $\alpha_1 \approx 0$  when the relation  $\epsilon_{11} \cdot \epsilon_{32} \approx \epsilon_{12} \cdot \epsilon_{31}$  exists purely by chance; thus, a statement is not possible about the number of components – independent of the course of  $\Delta A_2$ ).
- Straight lines running parallel to the coordinate axes are also degenerated (for example in the case of  $\epsilon_{11} = \epsilon_{21} = 0$ , the following equation is true



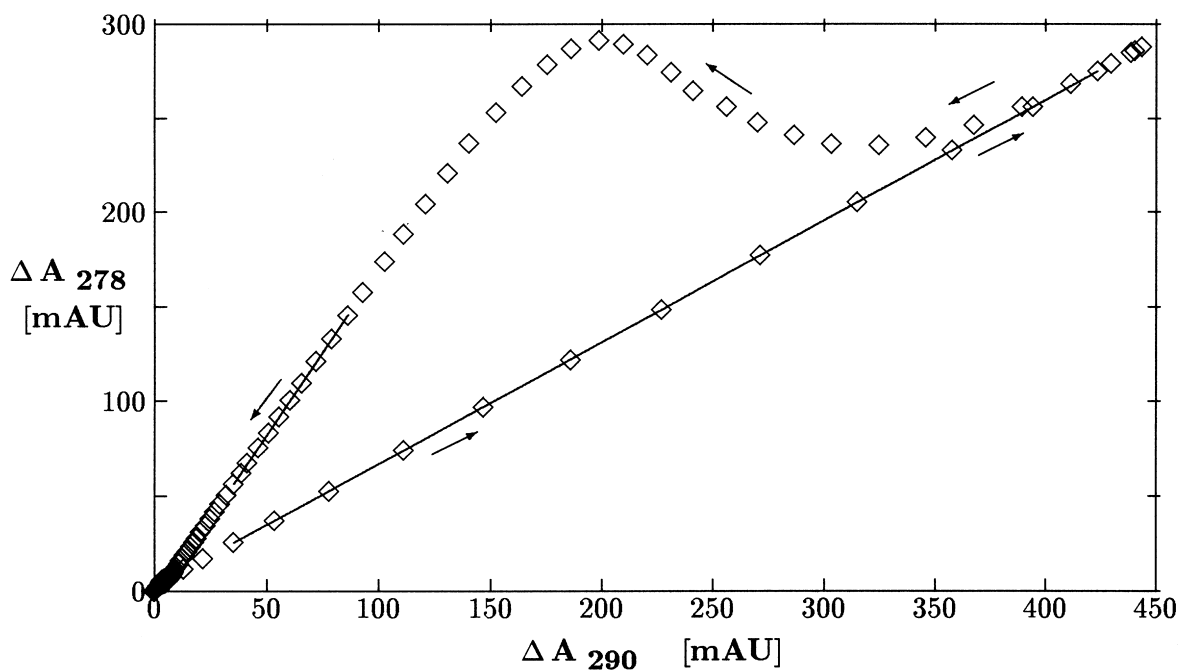


Fig. 3. AD diagram  $\Delta A_{278}$  vs.  $\Delta A_{290}$  obtained from Fig. 2.

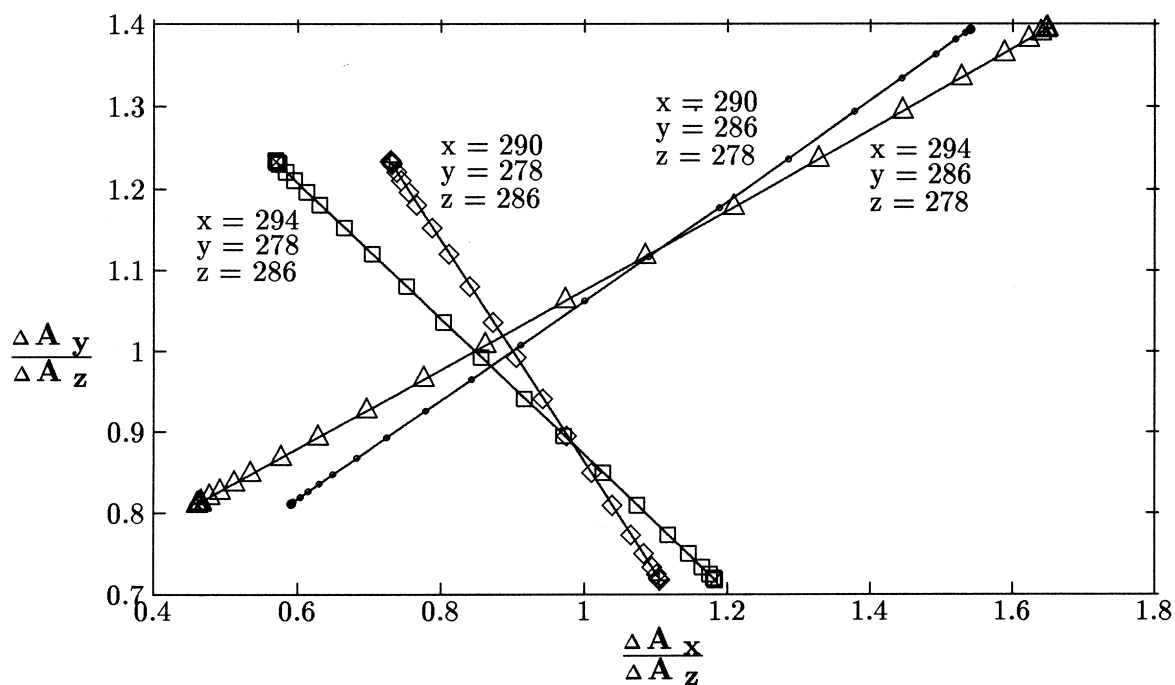


Fig. 4. ADQ diagram obtained from Fig. 2 ( $x, y, z$  declare the wavelengths used).

$(\Delta A_1)/(\Delta A_2) = (\epsilon_{12})/(\epsilon_{22})$ , which means that a parallel to the axis  $(\Delta A_3)/(\Delta A_4)$  is obtained being independent of the course of  $\Delta A_3$ .

The measured data in Fig. 2 has a high quality. The spectra show a high signal-to-noise ratio. Therefore, it is not necessary to construct integral A or ADQ diagrams from this data. However, when the spectroscopic quality of individual peaks is poor, integral or mean diagrams are helpful. The fluctuations of values to be evaluated are diminished by the introduction of integration or summation procedures. Furthermore, segments of curves created by these values are extended to that point where significance is improved. This is demonstrated in the last three diagrams.

The 'normal' ADQ diagram for the wavelengths 278, 286, 290 nm is shown in Fig. 5. The points of the mixture lie on a straight line. They form a more or less distinct segment on the corresponding line. The segment lies between two points which are produced by the 'pure' individual substances (chlorogenic acid and epicatechin).

The ADQ value  $(\Delta A_j)/(\Delta A_i)$  is the value of a slope belonging to a straight line of the corresponding AD diagram  $\Delta A_j$  vs.  $\Delta A_j$ . The correlation between AD and ADQ diagrams is explained with Fig. 3. The first points starting here from the origin lie on a straight line. This line has a special slope  $(\Delta A_{278})/(\Delta A_{290})$ . In the same way, the first points of the AD diagram  $(\Delta A_{286})/(\Delta A_{290})$  lead to another straight line with the slope  $(\Delta A_{286})/(\Delta A_{290})$ . The values of both slopes  $(\Delta A_{278})/(\Delta A_{290})$  and  $(\Delta A_{286})/(\Delta A_{290})$  are the coordinates of one point in the corresponding ADQ diagram (see 'first point 290 of evaluation' in Fig. 5). This point lies very close to the 'point of pure chlorogenic acid' which is produced by the slopes of the corresponding AD diagrams belonging to different concentrations of pure chlorogenic acid. Since this point seems to fall together with the 'first point of evaluation' a complete separation between both substrates was obviously achieved in the initial stage of the chromatographic process.

When all points, which do not lie on the initial

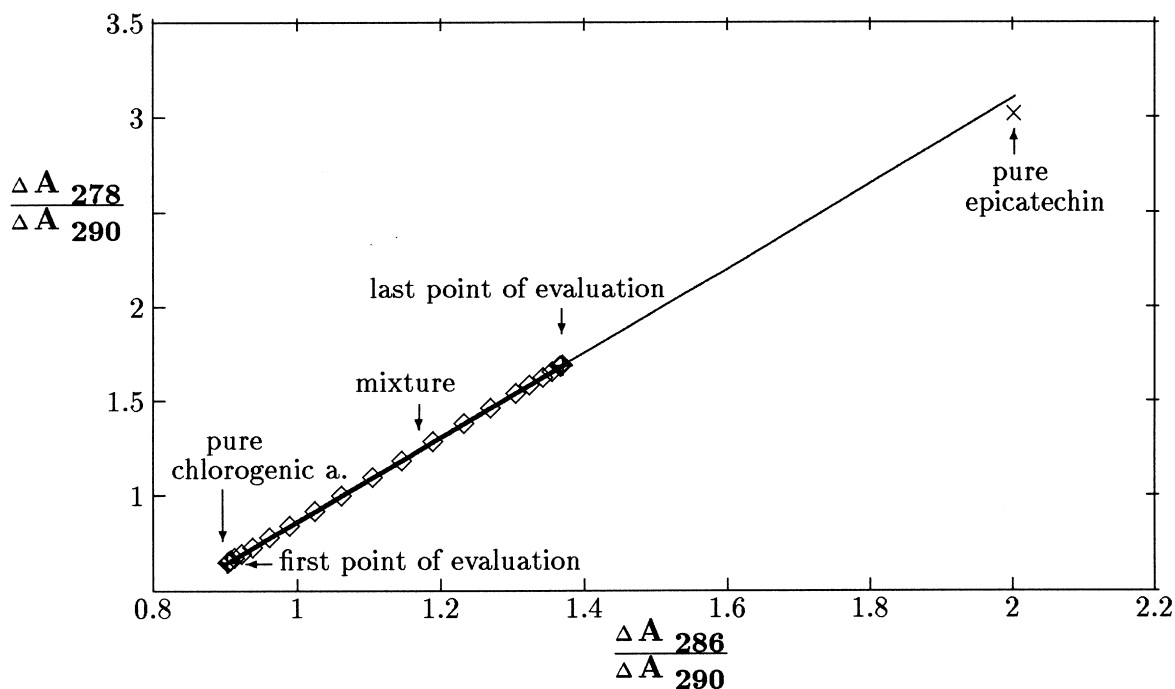


Fig. 5. ADQ diagram for 278, 286 and 290 nm of Fig. 2. The ADQ values of the pure substrates (chlorogenic acid and epicatechin) lie on a straight line created by the mixture, according to Eq. (10):  $\alpha_1 = -1.36$ ,  $\alpha_2 = 2.23$ , regression coefficient  $\approx 1.0$ .

straight line of Fig. 3, are connected with the origin, a series of straight lines with different slopes is obtained. These different slopes lead to different coordinates in the ADQ diagram and therefore to different ADQ points.

The ‘point of pure epicatechin’ is relatively far away from the ‘last point of evaluation’ (see Fig. 5). This means that both substrates were poorly separated within the incline of the 3D peak (see Fig. 2).

The geometric interpretation of the iADQ diagram is possible in the same way. The integral plot corresponding to Fig. 5 is shown in Fig. 6. The areas under the curves  $\Delta A$  vs.  $\lambda$  were simply determined by collecting the trapeze area produced by the individual  $\Delta A$  values and the corresponding wavelengths (or alternatively, by numerical routine procedures). The intervals of the wavelength range chosen for the integration amount here to 4 and 8 nm. Two distinct differences can be observed in comparison to Fig. 5:

- In Fig. 6 the ‘point of pure chlorogenic acid’ does

not fall exactly together with the ‘first point of evaluation’. Obviously the separation was not totally complete at the beginning of the chromatographic peak.

- The segment of straight line belonging to Fig. 6 is about 50% larger than that of Fig. 5. Therefore, a higher sensitivity is achieved in Fig. 6.

These facts demonstrate that the results can still be improved by integral plots. However, the choice of the optical bandwidth ( $\Delta\lambda = \lambda_j - \lambda_i$ ) is critical. If  $\Delta\lambda$  is too high, the significantly spectroscopic differences may be diminished between the single wavelength ranges and the evaluation becomes poorer. It seems that a range of 4–10 nm is optimal for many routine experiments.

The evaluation according to mADQ diagrams (Eq. (19)) leads to results which are similar to those obtained from the iADQ diagrams. In Fig. 7, the mADQ diagram which corresponds to the Figs. 5 and 6 is shown. Again, the ‘point of pure chlorogenic acid’ does not fall together with the ‘first point of

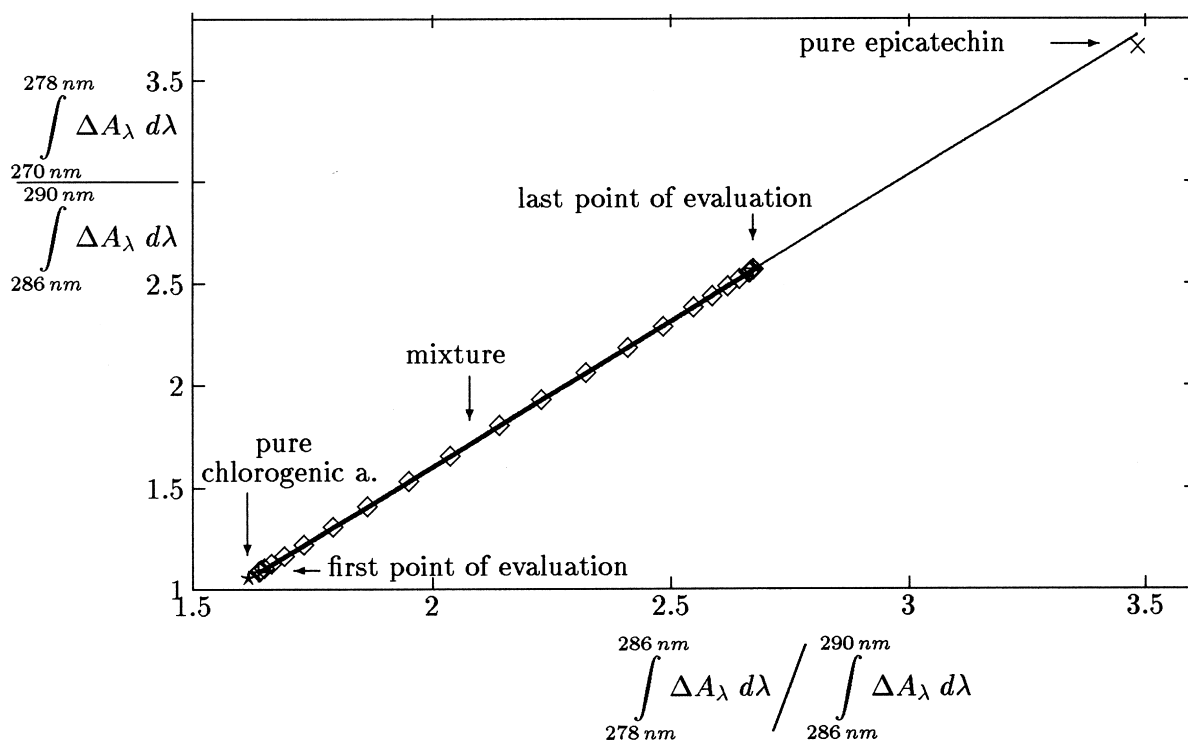


Fig. 6. iADQ diagram corresponding to Fig. 5 according to Eq. (15):  $\beta_1 = -1.25_2$ ,  $\beta_2 = 1.42_3$ , regression coefficient  $\approx 1.0$ .

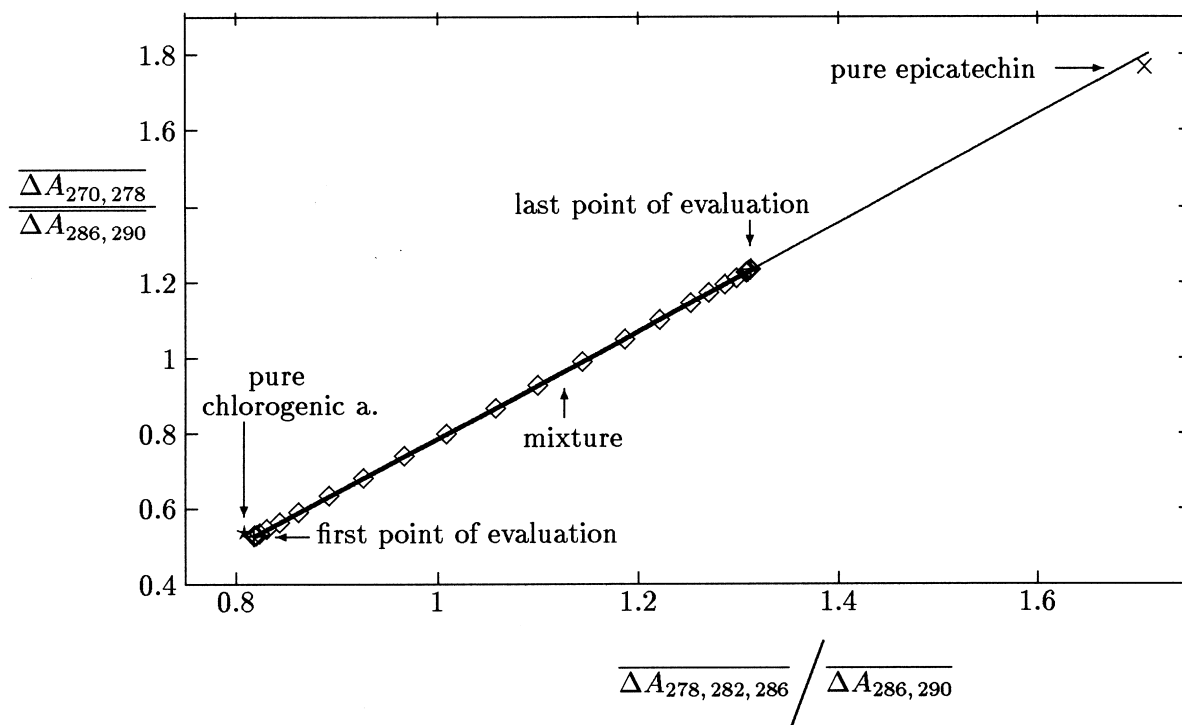


Fig. 7. mADQ diagram corresponding to Fig. 5, according to Eq. (19):  $\gamma_1 = -0.63_5$ ,  $\gamma_2 = 1.44_2$ , regression coefficient  $\approx 1.0$ ,  $\overline{\Delta A_{270,278}} = \frac{1}{2}(\Delta A_{270} + \Delta A_{278})$ ;  $\overline{\Delta A_{278,282,286}} = \frac{1}{3}(\Delta A_{278} + \Delta A_{282} + \Delta A_{286})$ ;  $\overline{\Delta A_{286,290}} = \frac{1}{2}(\Delta A_{286} + \Delta A_{290})$ .

evaluation'. However the segment of straight line belonging to Fig. 7 is not pronounced in such a way as in Fig. 6. Obviously, the most sensitive diagram is here in the example, the integral diagram followed by the mADQ and ADQ diagrams.

The mADQ diagram can be transferred to the corresponding iADQ diagram when the selected wavelengths lie closely together ( $\Delta\lambda \rightarrow 0$ , i.e.  $\Delta\lambda < 2$  nm). In Fig. 7, relatively high values for  $\Delta\lambda$  were chosen. Therefore, the numerical characteristics of the curves in the Figs. 6 and 7 are not identical (see  $\beta_i$  and  $\gamma_i$ ).

#### 4. Conclusions

The A and ADQ diagram established for the spectrometric analysis of reaction and titration systems can also be advantageously applied to peak purity analysis in chromatography. The construction of these plots is very simple. The number of com-

ponents which were detected in chromatographic peaks can be determined very precisely, in particular for peaks with one, two and three components. The knowledge about spectra or molar absorptivities of individual components is not necessary for the application of the presented methods. The new concept is also available for electrophoretic systems investigated spectrometrically.

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