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Purity assessment and resolution of tetracycline hydrochloride samples analysed using high-performance liquid chromatography with diode array detection

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Abstract

High-performance liquid chromatography with diode array detection (HPLC–DAD) was used for the analysis of a tetracycline hydrochloride sample (TC-HCl). The analytical search for impurities in TC-HCl is complicated, because the standards are not always available and because unexpected impurities are produced by new mutants during fermentation. Due to this it is useful to check the purity of the peaks in the chromatogram even when it is obtained with an optimised analytical technique. The homogeneity of the individual peaks in the HPLC–DAD chromatogram was checked using chemometric methods such as the orthogonal projection approach (OPA) and the fixed size moving window evolving factor analysis approach (FSW-EFA). After the selection of the number of components, the experimental data were resolved in the pure component spectra and the individual concentration profiles. The multivariate curve resolution alternating least-squares approach (MCR-ALS) was applied for this purpose on the single HPLC–DAD chromatograms (two-way data) and on sets of such chromatograms (three-way data).

Keywords: Purity assessment; Orthogonal projection approach; Fixed size moving window evolving factor analysis approach; Multivariate curve resolution alternating least-squares approach; Tetracyclines

1. Introduction

Tetracycline (TC) is an important broad-spectrum antibiotic, which is extensively used in human medicine. Tetracycline can contain several impurities from fermentation (such as 2-acetyl-2-decarboxamidotetracycline (ADTC), dimethyltetracycline (DMTC), chlortetracycline (CTC)) or from degradation such as 4-epitetracycline (ETC), 4-epianhydrotetracycline (EATC) and anhydrotetracycline (ATC). The structures of TC and its impurities are shown in Fig. 1. The determination of these impurities in commercial products is mainly performed by liquid chromatography (LC) and has been studied extensively [1–3]. In recent years, capillary electrophoresis (CE) for the analysis of tetracyclines has also been reported [4]. Micellar electrokinetic chromatography [5] has also been applied recently to study the migration behavior and the separation of tetracycline antibiotics. Thin-layer chromatographic (TLC) methods for rapid and simple control of the purity of tetracyclines have also been described [6,7]. The LC and CE methods use a classical one-wavelength detection and can be used to study the known

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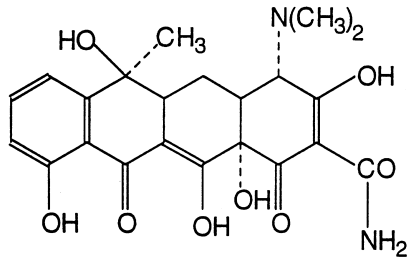
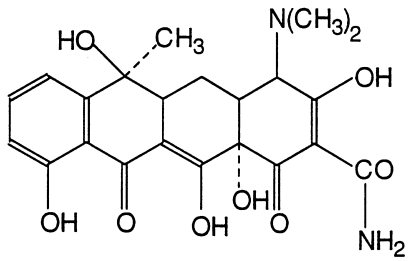
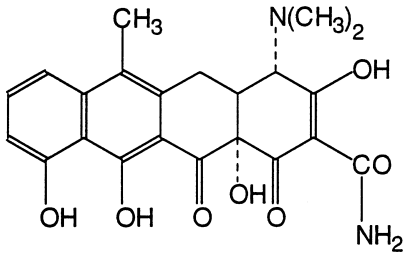


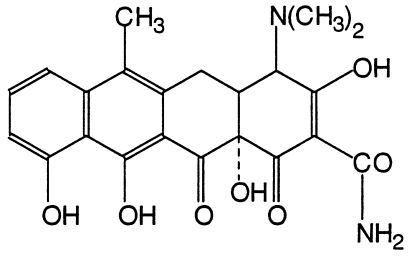
Tetracycline (TC)



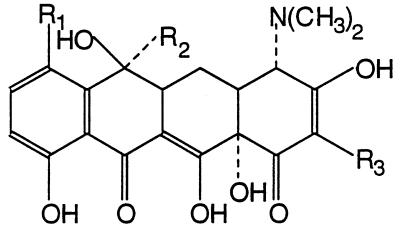
4-Epitetracycline (ETC)



Anhydrotetracycline (ATC)



4-Epianhydrotetracycline (EATC)



	R_1	R_2	R_3
Dimethyltetracycline (DMTC)	H	H	$CONH_2$
Chlortetracycline (CTC)	Cl	CH_3	$CONH_2$
2-acetyl-2-decarboxamidotetracycline (ADTC)	H	CH_3	$COCH_3$

Fig. 1. Structures of TC, ETC, ATC, EATC, DMTC, CTC and ADTC.

impurities. However, it is also important to check the purity of the chromatographic peaks to search for unexpected impurities produced by new mutants during the fermentation process. Data obtained with hyphenated techniques, such as high-performance liquid chromatography coupled with a diode array detector (HPLC–DAD), are analysed with chemometric methods such as the orthogonal projection approach (OPA) [8–11] and the fixed size moving window evolving factor analysis approach (FSW-EFA) [12–16] for this purpose. The goal of this work is to study to what extent chemometric methods are useful to detect and resolve known and unknown impurities in a TC-HCl sample. The chromatographic conditions of the HPLC method described in the USP XXIII [2] for the determination of tetracycline HCl (TC-HCl) were applied for the analysis of the TC-HCl sample. Ruggedness tests on the USP XXIII method [17,18] indicate that the pH of the mobile phase influences the separation of TC from its impurities. OPA and FSW-EFA were applied on data matrices recorded in cases where the separation power of the USP XXIII method becomes worse due to these pH changes. The resolution of the data matrices in the pure component spectra and the individual concentration profiles was performed with the multivariate curve resolution alternating least-squares approach (MCR-ALS) [19–23]. The resolution results obtained by the application of MCR-ALS on single data sets (two-way data) were compared with the results obtained with MCR-ALS applied on combined matrices (three-way data).

2. Experimental

2.1. Thin-layer chromatography (TLC)

2.1.1. Reagents and solutions

Disodium EDTA, sodiumhydroxide, dichloromethane and methanol pro analyse (E. Merck, Darmstadt, Germany) were used. Standards for TC-HCl (purity of 98.2%), ETC-HCl, EATC-HCl and ATC-HCl from Acros Chimica (Beerse, Belgium) were also available. The standards were dissolved in methanol in a concentration of 2 mg/ml. A TC-HCl sample which contains the following impurities 3% ETC-HCl, 2% EATC-HCl, 0.9% ADTC-HCl and 3% ATC-HCl was obtained from Professor Hoogmartens (Katholieke Universiteit Leuven, Belgium). The sample solution is a solution of 2 mg/ml sample mixture in methanol.

2.1.2. Procedure

The TLC method described in Ref. [7] was used. TLC was performed on 20×20 cm glass plates precoated with silica gel layers (Macherey-Nagel, Dören, Germany; # 809013). Before use, the plates were sprayed with a 10% (m/v) solution of disodium EDTA, adjusted to pH 8.0 with a 40% (m/v) solution of sodium hydroxide. The plates were dried in a horizontal position for at least 1 h at room temperature and then in an oven (110°C) for 1 h, shortly before use. The sample and standard solutions were applied (10 μl) to the same plate. The chromatographic chamber was equilibrated with the mobile phase, dichloromethane–methanol–water (59:35:6) for at least 1 h prior to use. The plate was developed at room temperature over a distance of 15 cm. The detection of the components was performed under UV light at 365 nm.

2.2. High-performance liquid chromatography with diode array detection (HPLC–DAD)

2.2.1. Reagents and solutions

Potassium nitrate, ammonium oxalate, diammonium hydrogenphosphate, *N,N*-dimethylformamide (DMF) pro analyse (E. Merck, Darmstadt, Germany) were used. A TC-HCl sample which contains besides TC-HCl also 3% ETC-HCl, 2% EATC-HCl, 0.9% ADTC-HCl and 3% ATC-HCl obtained from Professor Hoogmartens (Katholieke Universiteit Leuven, Belgium) was used for the preparation of the sample solution. The solvent consists of ammonium oxalate 0.1 *M* and DMF (680:270). Potassium nitrate (1 mg/ml) was added in the solvent to determine the dead time. The TC-HCl sample was dissolved in the solvent in a concentration of 0.5 mg/ml.

Pure standards for TC and ADTC were not available and, therefore, they were obtained from the spots separated in the TLC plate. ADTC and TC were eluted from the silica gel with 1 ml methanol using a solid-phase extraction tube. The eluent was diluted with 3 ml water.

2.2.2. Instrument

An LC-10AD Shimadzu liquid chromatograph coupled with an SPD-M10A Shimadzu diode array detector was used for the analysis of the TC-HCl sample. The experiments were performed at a constant temperature of 30°C using an SPD-M10A Shimadzu column oven.

2.2.3. Chromatographic conditions

The chromatographic conditions described in the USP XXIII method [2] for the determination of TC-HCl were used. The column used is an Alltech Alltima C8 5 μm type with an internal diameter of 4.6 mm and a length of 25 cm. The mobile phase consists of ammonium oxalate 0.1 *M*, DMF and dibasic ammonium phosphate 0.2 *M* (680:270:50). The USP XXIII method states that the nominal pH is adjusted between 7.6 and 7.7. Slightly smaller pH values (7.50, 7.40, 7.20 and 7.00) were also tested. The pH was adjusted with 3 *N* ammonium hydroxide or 3 *N* phosphoric acid depending on the initial pH of the solution. The pH was measured with a WTW Microprocessor pH meter. The mobile phase was filtered through a filter with a porosity of maximum 0.5 μm and degasified on an ultrasonic bath (at least 5 min). The flow of the mobile phase was set on 1 ml/min and the volume injected was 20 μl. The USP XXIII method records only one chromatogram at wavelength 280 nm. In this article, the detection was performed in the wavelength region 250–400 nm, which yields data matrices. The data sets were stored using a bandwidth of 1 nm and a time constant of 0.24 s. The bandwidth [24] can be defined as the wavelength interval in which the chromatograms recorded are averaged. The time constant [24] is the time region in which the spectra are averaged. Large values for these parameters reduce the noise in the chromatographic and the spectral profiles, but also the ability to detect small differences. Since our goal is to detect compounds with small differences in both chromatographic and spectral directions we chose the smallest values for both parameters.

3. Data sets

Five data sets were obtained for the analysis of the TC-HCl sample with the HPLC–DAD instrument. Matrix M765 was recorded in the nominal conditions of the USP XXIII method at a pH of 7.65. Matrices M750, M740, M720 and M700 were measured at pH 7.50, 7.40, 7.20 and 7.00, respectively. The impurity ATC-HCl is well separated from the other components in the studied pH region. ATC-HCl is, therefore, removed from the recorded data sets before multivariate methods are applied.

Matrices ADTC_TLC and TC_TLC are those coming from the ADTC and TC spots observed on the TLC plate and analysed with HPLC–DAD in the nominal conditions of the USP XXIII method.

4. Data treatment

Each HPLC–DAD run yields a data matrix **X** ($m \times n$) where the m rows are spectra measured at regular time intervals and the n columns are chromatograms measured at different wavelengths. Multivariate mixture analysis methods like the OPA [8–11] and FSW-EFA [12–16] are commonly used on such data sets to assess the purity of substances. The MCR-ALS [19–23] can be applied to resolve the matrix in the pure component spectra and their corresponding concentration profiles.

4.1. Fixed size moving window evolving factor analysis (FSW-EFA)

Starting from the first spectrum, a window containing p consecutive spectra is moved along the data matrix **X**. If, e.g., $p=7$, the first window contains the seven first spectra, the second window spectra two to eight and so on. Each window is decomposed by means of singular value decomposition (SVD) and the logarithm of the p singular values are plotted as a function of the window number. The window size should be at least equal to the number of eluting compounds +1, though slightly larger windows have proven to be more operational to detect small impurities. The information provided by a FSW-EFA plot for a HPLC–DAD data set is a local rank map of the elution process, i.e., a plot that indicates the number of components eluting simultaneously in each time window analysed. The presence of a new eluting component is marked by the presence of a new singular value significantly

higher than those related to noise, which have similar numerical values and appear together at the bottom of the FSW-EFA plot. Relating this information to the problem of peak purity, pure elution regions will be identified with time windows where only the first singular value differs significantly from the noise level. Windows with two significant singular values will be related to elution regions where two components elute simultaneously and, in general, windows with p singular values different from the noise level point out regions where p components elute together. Nevertheless, in normal chromatographic processes, where fairly good separation conditions are used, the most common situation is finding regions with no significant components (related to noise), with one significant component (pure elution regions) or with two significant components (time windows with two components overlapping). Windows with two significant components mark time regions where either a partial or a complete overlap between two peaks occurs; this means that the presence of impurities completely embedded under a main peak can be detected.

4.2. The orthogonal projection approach (OPA)

Impurities are chemically related to the substance of interest and their spectra can, therefore, be very similar. Selective spectral regions are thus not always present in a HPLC–DAD data matrix **X**. However, chromatographic regions can exist where only one component elutes. OPA is, therefore, applied in the time direction of the matrix **X** to search for the most dissimilar spectra.

The dissimilarity is defined as the determinant of the dispersion matrix $\mathbf{Y_i} \times \mathbf{Y_i}'$ of $\mathbf{Y_i}$. Each matrix $\mathbf{Y_i}$ contains one or more reference spectra in addition to the measured spectrum $\mathbf{x_i}$. Initially, when no spectrum has been selected, the mean spectrum of **X**, $\mathbf{x_m}$, is considered as the reference in $\mathbf{Y_i}$. The mean spectrum and in general all reference spectra in $\mathbf{Y_i}$ are normalised to length equal to 1. The dissimilarity for each spectrum $\mathbf{x_i}$ with respect to the mean spectrum, $\mathbf{x_m}$, is plotted versus the corresponding retention time in the first dissimilarity plot. The spectrum with the highest dissimilarity is selected from this plot and is used, instead of the mean spectrum, as a reference in the matrix $\mathbf{Y_i}$ to calculate the second dissimilarity plot. The spectrum with the highest dissimilarity is selected in the second plot and added as a reference in the matrix $\mathbf{Y_i}$. The matrix $\mathbf{Y_i}$ contains now two reference spectra: the first and second selected spectrum. The process of selecting spectra with the highest dissimilarity in the successive dissimilarity plots and including them as additional references in the matrix $\mathbf{Y_i}$ is repeated as long as there are spectral features present in the plots. The number of spectra selected, equals the number of components present in the matrix **X**. The selected spectra, provide information about the spectra of the pure components.

4.3. Multivariate curve resolution alternating least-squares approach (MCR-ALS)

MCR-ALS is applied, after the selection of the number of components, to resolve the data matrix **X** into the pure component spectra and their related individual concentration profiles. The matrix **X** can be resolved individually (two-way data), can be combined with other chromatographic runs of the same mixture or with standards of the components present in the mixture data matrix (three-way data). No need of synchronisation in the time direction among the different runs is required. The two-way data set **X** can be written as

$$\mathbf{X} = \mathbf{CS}' + \mathbf{E} \tag{1}$$

where **C** is the matrix of the elution profiles, $\mathbf{S}'$ the matrix of the pure component spectra and **E** is the matrix which contains the residual absorption not caused by the components considered. The three-way data matrix is built by setting the matrices $\mathbf{X_k}$ (**k** being the number of the chromatographic run) one on top of each other, keeping a common wavelength range (columns) for all of them:

$$\mathbf{X} = \begin{bmatrix} \mathbf{X}_1 \\ \mathbf{X}_2 \\ \dots \\ \dots \\ \mathbf{X_k} \end{bmatrix} = \begin{bmatrix} \mathbf{C}_1 \\ \mathbf{C}_2 \\ \dots \\ \dots \\ \mathbf{C_k} \end{bmatrix} \mathbf{S}' + \mathbf{E} \tag{2}$$

The augmented data matrix **X** has a number of rows equal to the total number of acquired spectra in the different chromatographic runs. The new augmented

matrix is the product of an augmented concentration matrix times the pure component spectra matrix. The augmented concentration matrix contains the submatrices $\mathbf{C_k}$ corresponding to the elution of the components present in each of the data matrices $\mathbf{X_k}$ analysed. As before, $\mathbf{S}'$ contains the pure component spectra and $\mathbf{E}$ the residual absorption.

Once the two-way or three-way data set is built, the decomposition of the $\mathbf{X}$ matrix takes place following the next steps:

4.3.1. Building a matrix of initial estimates

To start the resolution process, a matrix of initial estimates, either containing concentration profiles or spectra, is necessary. These estimates are easily built by using data analysis methods, such as evolving factor analysis (EFA) [25–29], OPA or many others.

For three-way data sets, the augmented matrix of concentration initial estimates, $\mathbf{C}$, is built by placing the initial estimates related to each chromatographic run, $\mathbf{C_k}$, one on top of each other sorted as the $\mathbf{X_k}$ matrices are to form $\mathbf{X}$.

4.3.2. Application of the multivariate curve resolution alternating least-squares (MCR-ALS) optimisation to obtain the definitive concentration and spectra matrices

The equation $\mathbf{X}=\mathbf{CS}'$ is solved iteratively by using a constrained least-squares procedure. The resolution process goes to the final solution by updating the spectra and the concentration profiles obtained by least-squares in each iterative cycle according to a series of selected constraints. These constraints are related either to the internal structure of the data set or to the chemical features of the experimental measurements.

In the resolution of the presented HPLC–DAD data, the constraints applied to two- or three-way data sets were selectivity in the chromatographic direction (i.e., keep fixed the time windows where only one component elutes by setting the absent species, concentrations or absorbances, equal to zero), non-negativity in the concentration profiles and spectra and unimodality (i.e., only one maximum is allowed) in the chromatographic profiles.

When working with three-way data sets, no constraints forcing the peak shape and position of each eluted compound to be the same in all chromatographic runs have been applied. In contrast, only one pure spectrum per compound has been postulated because there are no spectral variations in the range of chromatographic conditions used in the different analysed runs.

The quality of the MCR-ALS results is evaluated by calculating the lack of fit with respect to the experimental data (exp) and the principal component reconstructed data (PCA) [30], expressed as follows:

$$\%\ \text{lack of fit} = \sqrt{\frac{\sum_{ij} (x_{ij} - x_{ij}^{*})^2}{\sum_{ij} x_{ij}^2}} \times 100 \qquad (3)$$

where x_{ij} are the experimental data or the PCA reconstructed data of the HPLC–DAD data set and x_{ij}* the reproduced data by using the MCR-ALS method. Subscripts i and j are referred to rows and columns of the original data matrix, respectively.

The MCR-ALS procedure is repeated until a predetermined number of cycles have occurred or until convergence is achieved. Convergence is achieved if the relative change in the lack of fit between consecutive iterations becomes smaller than the convergence limit defined by the user. If the relative change is negative and/or its absolute value is larger than the convergence limit, the optimisation is not correct, and the process is diverging in this case. The convergence limit used in this article is 0.1%.

Working with three-way data sets introduces a great decrease of the ambiguity associated with the decomposition of bilinear matrices [20,21] due to the use of additional constraints and the use of the information included in better resolved matrices into the resolution of matrices showing worse conditions of overlap among components.

5. Results and discussion

5.1. Purity assessment

5.1.1. Purity assessment with TLC

The presence of ETC, EATC, TC, ATC and ADTC in the TC-HCl sample was confirmed by TLC [7]. The components ETC, TC and ADTC showed a

green–yellowish fluorescence under UV light of 365 nm, while EATC and ATC gave an orange fluorescence. The components TC and ADTC were isolated from the TLC plate and analysed with HPLC–DAD. The HPLC–DAD results show that ADTC and the main component TC have very similar retention times. The presence of ADTC is only assumed by Ref. [7], but there is no structural result that supports this theory. Despite this, it is confirmed that the main component and an impurity elute with a small chromatographic resolution. This impurity will be indicated as ADTC in this article.

5.1.2. Purity assessment with FSW-EFA

The application of FSW-EFA results in the singular value plots given in the Fig. 2a–d for the matrices M765, M740, M720 and M700, respectively. A size seven window was used. The singular value plot for matrix M750 was analogous with the plot obtained for matrix M765. Therefore, it is not shown in Fig. 2.

Fig. 2a represents the matrix M765. The shape of the first singular value plot recalls the mean chromatogram and clearly shows the presence of ETC, EATC, TC and two unknown impurities labelled unknown1 and unknown2. The unknown interferences are not detected with TLC, probably due to their low concentration. Zones where the second singular value arises from the noise level indicate the overlap regions between neighbouring peaks. The impurity ADTC is not observed under TC. The five last singular value plots have the same shape and are related to noise contributions. The sudden increase under the TC peak points to the presence of heteroscedastic noise. The FSW-EFA results for the matrix,

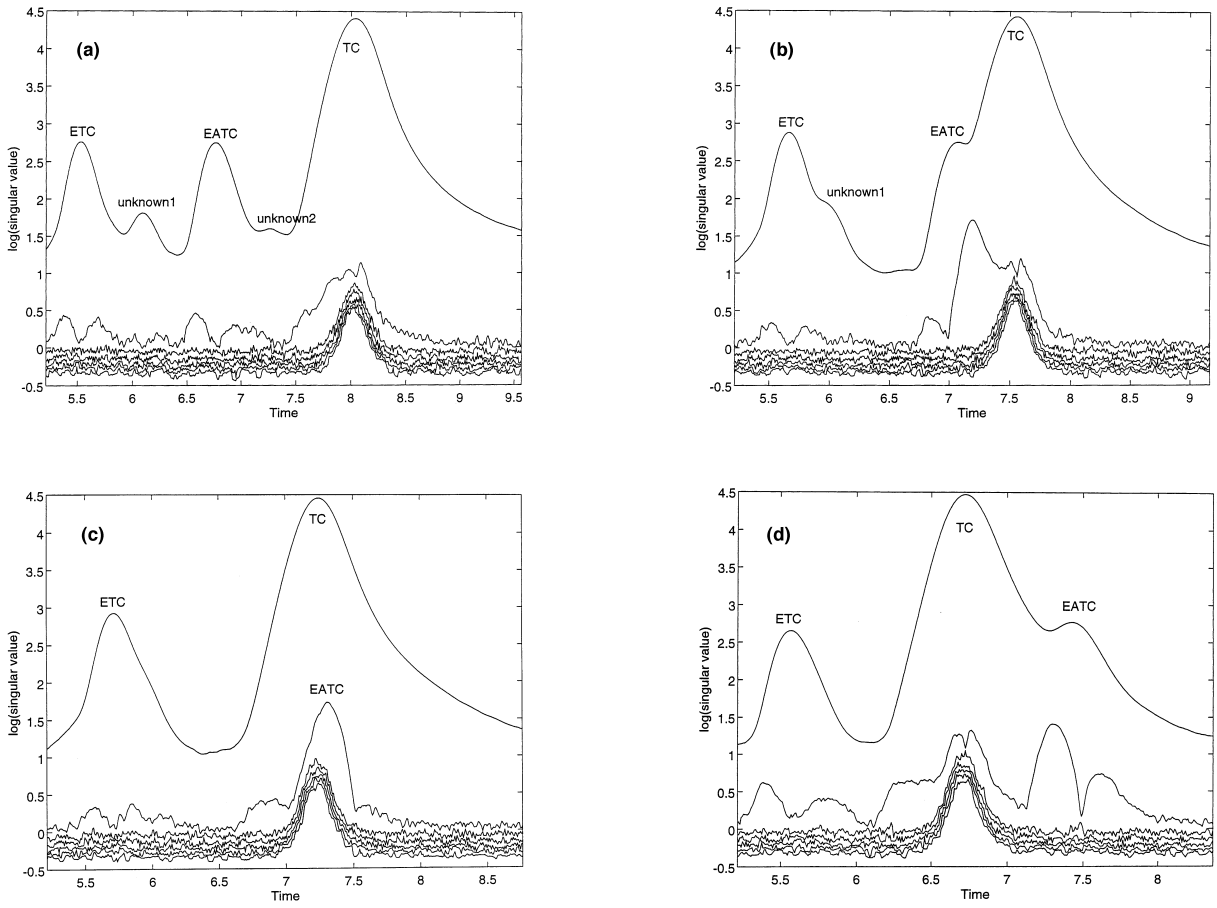


Fig. 2. FSW-EFA plots for the matrices M765 (a), M740 (b), M720 (c) and M700 (d).

measured at pH 7.50, are comparable with the results obtained for the matrix M765. The impurity between EATC and TC is not detected in this matrix with FSW-EFA. Fig. 2b shows the results for pH 7.40. The plot clearly shows the presence of ETC, unknown1, EATC and TC in the first singular value plot. The second singular value plot shows the time region where EATC and TC overlap. The presence of ADTC is also not detected. The plot in Fig. 2c shows that at pH 7.20 EATC is completely covered by TC and that the peak maxima of both components are very similar. This is clearly observed in the second singular value plot. The plot for matrix M700 (see Fig. 2d) indicates that the elution order of the components TC and EATC is switched.

5.1.3. Purity assessment with OPA

5.1.3.1. Purity analysis of TC and ADTC standards isolated with TLC and analysed with HPLC–DAD

The OPA dissimilarity plots and the selected retention times were compared for the ADTC_TLC and TC_TLC data sets.

OPA selects two spectra in the matrix TC_TLC at the times 8.576 and 8.992 in the first and second dissimilarity plot, respectively (see Fig. 3a,b). The third plot (see Fig. 3c) shows a random structure of low dissimilarity values and represents only noise. The spectrum measured at time 8.576 represents TC. The spectrum at time 8.992 is probably due to degradation of TC during the TLC method, because the stationary phase in the TLC method is sprayed with a disodium EDTA solution adjusted to pH 8.0. TC degrades due to this alkaline pH because of the presence of a phenol function in its structure (see Fig. 1). This reaction probably occurs when the migration-speed decreases and the interaction between TC and the stationary phase increases. Due to this, TC and its degradation product are not separated with the TLC method. The mean chromatogram obtained for the HPLC–DAD data set shows a small shoulder for the degradation product. OPA clearly shows the presence of both components.

The dissimilarity plots obtained for the matrix ADTC_TLC are identical with the plots obtained for matrix TC_TLC. The spectra at the times 7.968 and 8.160 are selected for ADTC and its corresponding degradation product. The mean chromatogram of the recorded HPLC–DAD data set shows only one peak. Nevertheless, both components are clearly detected with OPA.

The times 8.576 and 7.968 selected with OPA in the matrices TC_TLC and ADTC_TLC for TC and ADTC, respectively, indicate that both components elute with a low chromatographic resolution in the nominal conditions of the USP XXIII method.

5.1.3.2. Purity analysis of TC-HCl sample analysed at the pH of 7.65

The dissimilarity plots obtained with OPA for the matrix M765 are shown in Fig. 4a–e. The spectra recorded at the times 8.032, 6.76, 5.528 and 7.896 are selected for TC, EATC, ETC and ADTC, respectively. Fig. 4a,b shows that after the selection of the spectrum for TC in Fig. 4a two peaks appear, one at each side of the spectrum selected for TC (see Fig. 4b). These peaks become more dominant in the plots 4c,d. One of these peaks is selected for ADTC in plot 4d. The plot in Fig. 4e shows very small dissimilarity values compared with the plot in Fig. 4d. Therefore, it was decided that this plot represents only heteroscedastic noise. The two peaks, which appear in the region of the main component (TC), could also be interpreted as a non-linearity artefact [8] in the measurements, if OPA had been applied on the data sets without prior information about the sample. In this case, the presence of ADTC was confirmed by TLC. The peaks are, therefore, attributed to ADTC. The unknown impurities, observed in the first singular value plot of Fig. 2a, are not detected when OPA is applied on the matrix M765. The application of OPA in the time regions where the unknown impurities (unknown1 and 2) elute did not indicate their presence either. This is due to the fact that both impurities are not significantly different from the noise level.

5.1.3.3. Purity analysis of TC-HCl samples analysed at pH 7.50, 7.40, 7.20 and 7.00

The interpretation of the dissimilarity plots obtained for matrices M750, M740 and M700 is analogous with the results shown in Fig. 4a–e for the matrix M765. For the matrix M720 the OPA dissimilarity plots in Fig. 5a,b clearly shows the presence of EATC under the TC peak. The decrease in the dissimilarity is used as a criterion to select the

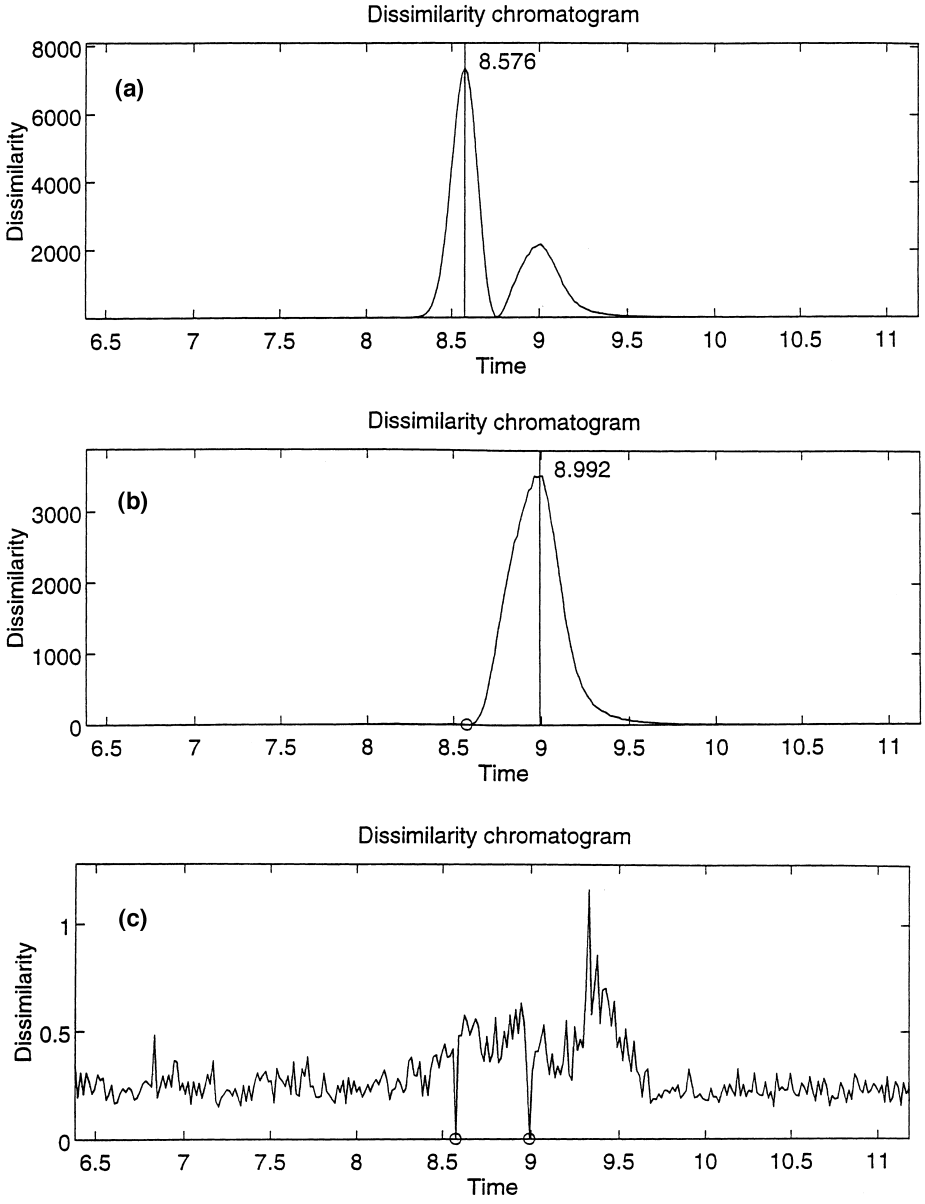


Fig. 3. OPA results from the analysis of TC_TLC matrix. Dissimilarity plots.

component ADTC in the matrices M750, M740, M720 and M700. The times selected with OPA for the components ETC, EATC, ADTC and TC are listed in Table 1 for matrices M765, M750, M740, M720 and M700. These times mark the peak maxima of the components ETC, EATC, ADTC and TC. Table 1 shows that the retention times for ADTC and TC are very similar.

5.2. Resolution with MCR-ALS

The resolution of the data matrices into the pure

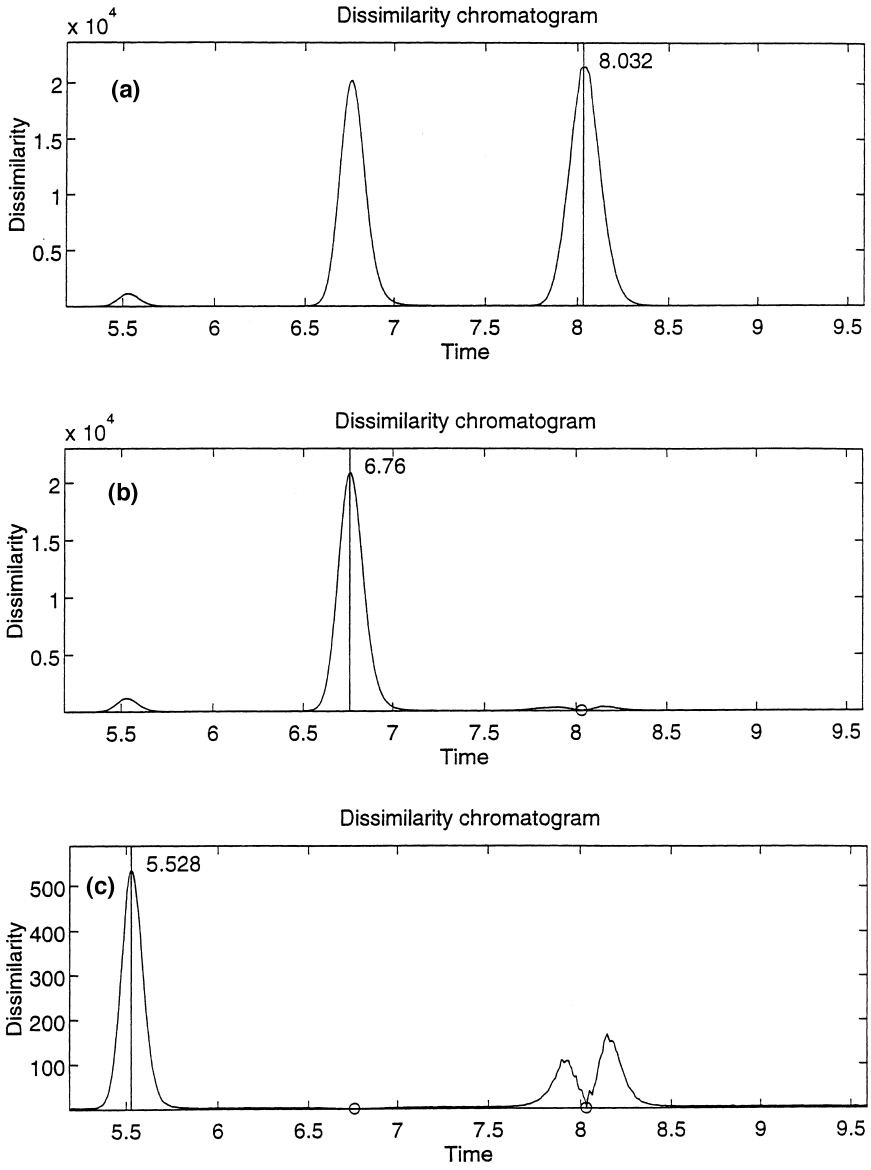


Fig. 4. OPA results from the analysis of the matrix M765. Dissimilarity plots.

component spectra and the individual concentration profiles is performed with MCR-ALS, after the selection of the number of components with OPA. MCR-ALS can be performed using spectral or concentration initial estimates for the selected number of components. For the resolution of partially overlapped chromatographic components, concentration initial estimates are preferred. In this case, where the components ADTC and TC have a complete overlap in the chromatographic direction, spectral initial estimates were also used. MCR-ALS was applied to the single data matrices (two-way data) and to multiple chromatographic runs (three-way data), using spectra and concentration profiles as initial estimates. The resolved spectra, concentration profiles and the lack of fit of the resolved data with

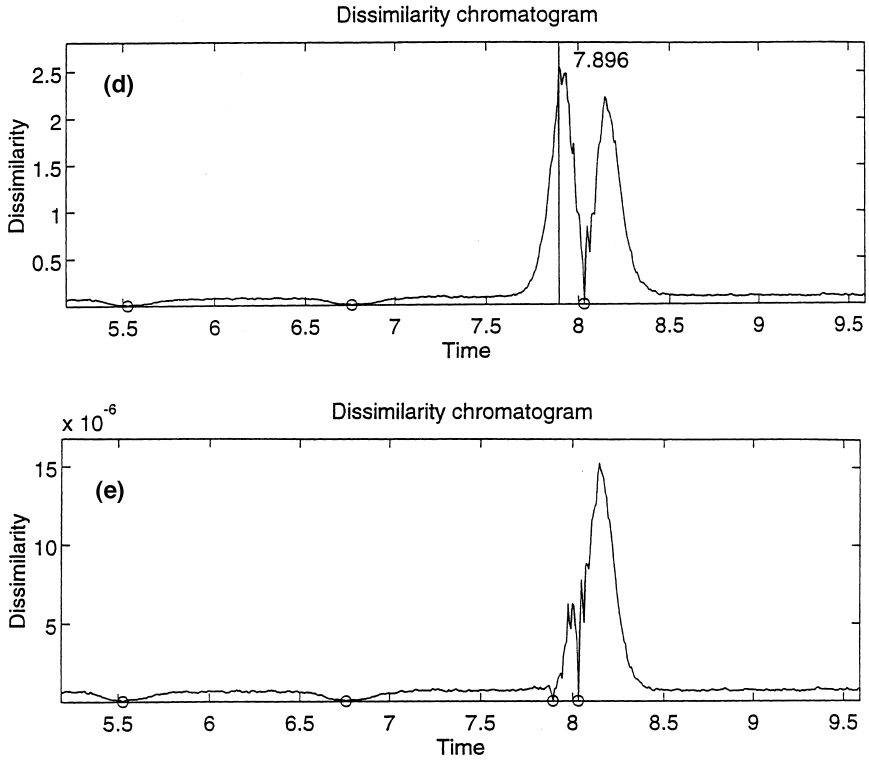


Fig. 4. (*continued*)

respect to the PCA reconstructed data (PCA) and the experimental data (exp) were used to evaluate the resolution results.

5.2.1. MCR-ALS applied to two-way data

The matrices ADTC_TLC, TC_TLC, M765, M750, M740, M720 and M700 were resolved with MCR-ALS, using the spectra selected with OPA as initial estimates. The spectra or times selected with OPA in the matrices M765 and M750 for the components ETC, EATC, ADTC and TC are very similar (see Table 1) and, consequently, the resolved spectra and concentration profiles do not show important differences between them. Due to this, the resolution results for matrix M750 will not be further discussed. The resolved spectra and concentration profiles for the matrices M765, M740, M720 and M700, using spectral initial estimates, are shown in Fig. 6a–d, respectively. MCR-ALS was also applied using concentration initial estimates, which were obtained from the individual resolution of the data matrices, using the spectra selected with OPA. The fitting errors (listed in Table 2) are usually small and show that the resolution results are comparable when spectra or concentration profiles are used as initial estimates. Table 2 also indicates that the number of iterations needed to reach the convergence criterion is much lower when concentration initial estimates are used, which enhances the calculation speed. The optimisation of matrix TC_TLC was stopped before divergence appeared. Nevertheless, the small fitting errors and the resolved profiles indicate that the resolution of matrix TC_TLC is successful. The somewhat higher fitting error for ADTC_TLC matrix is due to the very low concentration of the standard in this matrix. In this case, the signal to noise ratio is much less favourable than for the other matrices. The spectra obtained from the individual resolution of ADTC_TLC and TC_TLC for components ADTC and TC are presented in Fig. 7. Both spectra are very similar in the studied wavelength range (correlation coefficient, 0.9941). For the mixture matrices M765,

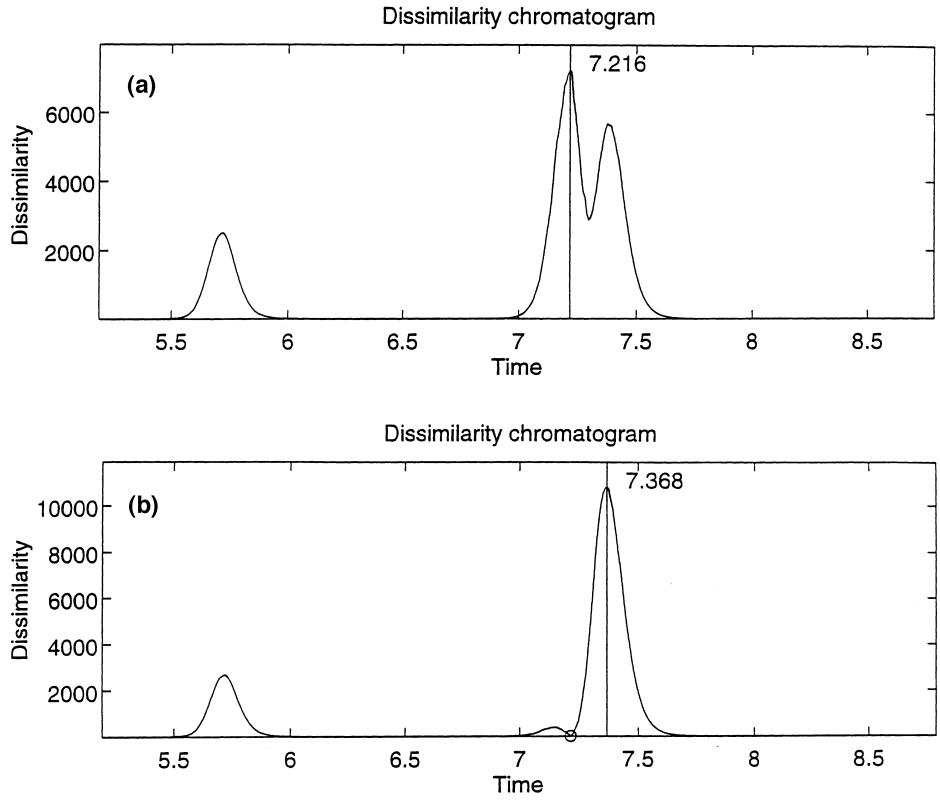


Fig. 5. OPA results from the analysis of the matrix M720. First and second dissimilarity plot.

M740 and M700, the spectra recovered for ETC, EATC and TC are quite similar and their concentration profiles present the magnitude and elution sequence expected. In contrast, due to the high overlap between TC and ADTC in both chromatographic and spectral directions, and to the very low proportion of ADTC in the samples (even lower than the other impurities), the ADTC spectrum is generally badly recovered and the concentration profile related to this compound, though well located, does not always present the expected low proportion with respect to the main compound, TC (see M740 results, Fig. 6b). The high chromatographic overlap between EATC, ADTC and TC at pH 7.20 also results in badly recovered concentration profiles for these components. The spectrum resolved for EATC does not have the expected shape. The fitting errors of the resolution results for matrix M720 (Table 2) indicate that the resolution is somewhat less favourable than for the matrices M765, M740 and M700. These ambiguities in the recovery of the profiles are typically associated with the use of resolution methods for individual matrices with severe overlap between compounds [31].

Table 1
Times selected with OPA for the components ETC, EATC, ADTC and TC in the matrices M765, M750, M740, M720 and M700

Matrix	ETC	EATC	ADTC	TC
M765	5.528	6.76	7.896	8.032
M750	5.624	6.928	7.944	7.816
M740	5.664	7.056	7.464	7.552
M720	5.712	7.368	7.136	7.216
M700	5.560	7.440	6.624	6.728

5.2.2. MCR-ALS applied to three-way data

The matrices M765, M740, M720 and M700 were each combined with the matrices ADTC_TLC and TC_TLC to build three-way data sets of the form:

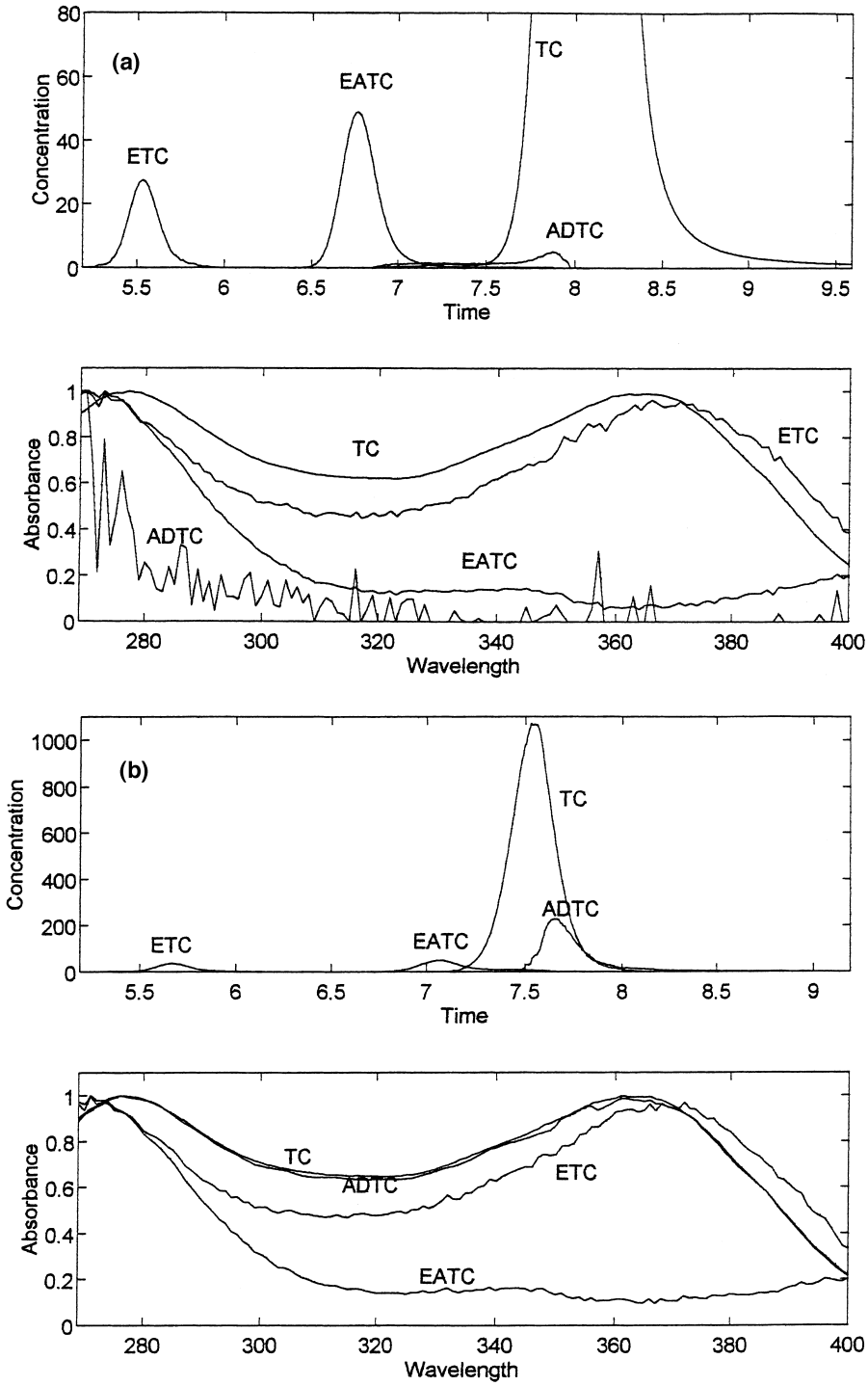


Fig. 6. Resolved concentration profiles and spectra for the individual resolution of matrices M765 (a), M740 (b), M720 (c) and M700 (d), using MCR-ALS and spectral initial estimates.

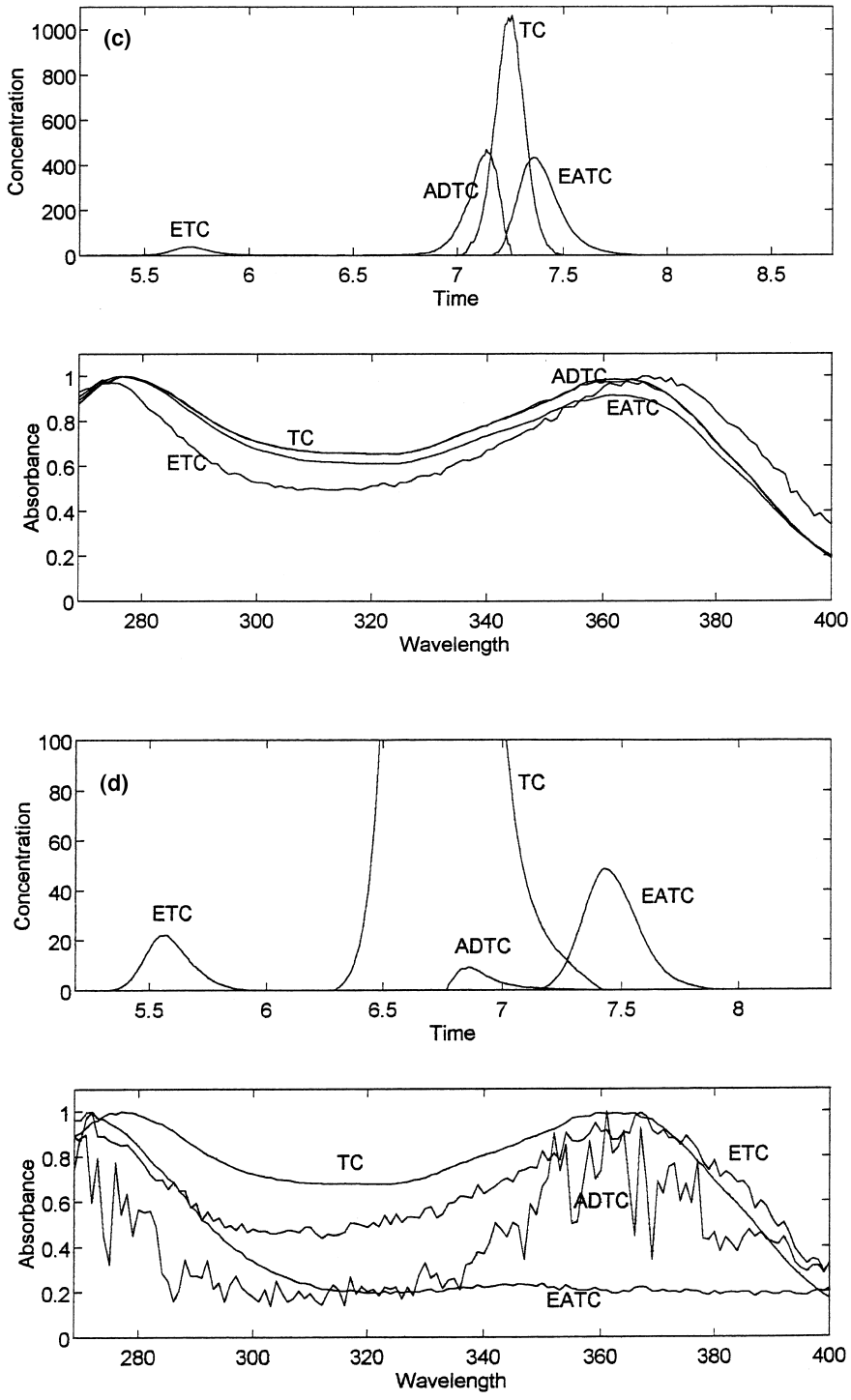


Fig. 6. (*continued*)

Table 2
Fitting error of the individual resolution results with respect to the PCA reconstructed data (PCA) and the experimental data (exp) and the number of iterations to reach the convergence criterion (0.1%)

Matrix	Fitting error: individual resolution					
	Spectral initial estimates			Concentration initial estimates		
	PCA	Exp	Iteration	PCA	Exp	Iteration
ADTC_TLC	2.106	3.170	17	2.103	3.168	2
TC_TLC	1.478	1.624	—[a]	1.484	1.629	—[a]
M765	0.529	0.544	46	0.526	0.541	4
M740	0.323	0.347	28	0.289	0.316	14
M720	2.126	2.128	46	1.294	1.298	7
M700	0.509	0.526	47	0.509	0.526	10

[a]Solutions obtained before divergence appears.

$$\begin{bmatrix} M_x \\ \text{ADTC\_TLC} \\ \text{TC\_TLC} \end{bmatrix}$$

where M_x refers to one of the HPLC–DAD matrices recorded for the TC-HCl sample at a specified pH value x. The three-way data sets were resolved using the spectra selected for ETC and EATC with OPA in the two-way matrices M765, M740, M720 and M700. The spectral estimates for ADTC and TC were obtained with OPA from the matrices ADTC_TLC and TC_TLC, respectively. The simultaneous analysis of the mixture data set with the ADTC_TLC and TC_TLC data matrices results in a better spectrum for ADTC in matrix M765 (see Fig. 8a) and a concentration profile located where it was expected. The two additional spectra are those of the degradation products for ADTC and TC present in TC_TLC and ADTC_TLC data matrices. The results for M740 are also improved (see Fig. 8b). The concentration profile of ADTC has the expected retention time and peak area. The spectrum for

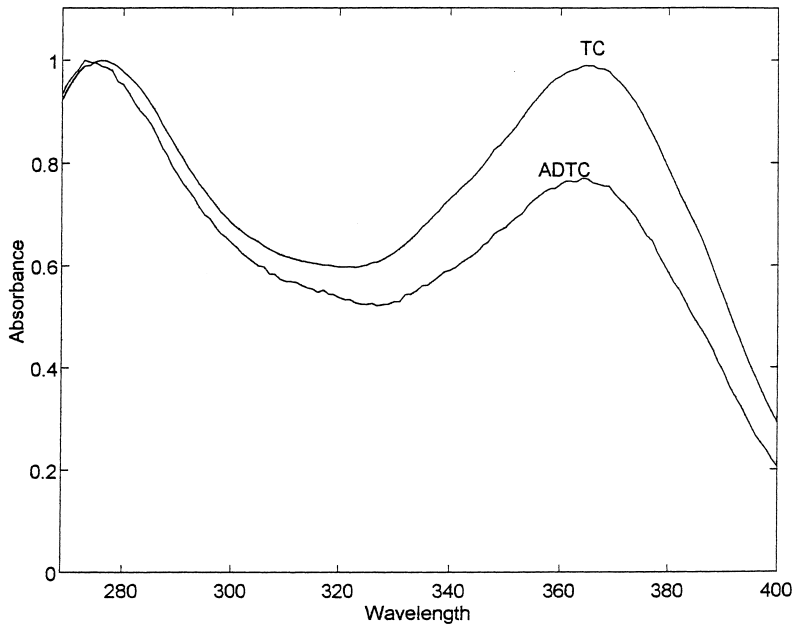


Fig. 7. Recovered spectra of TC and ADTC after application of MCR-ALS to the individual TC and ADTC standard matrices.

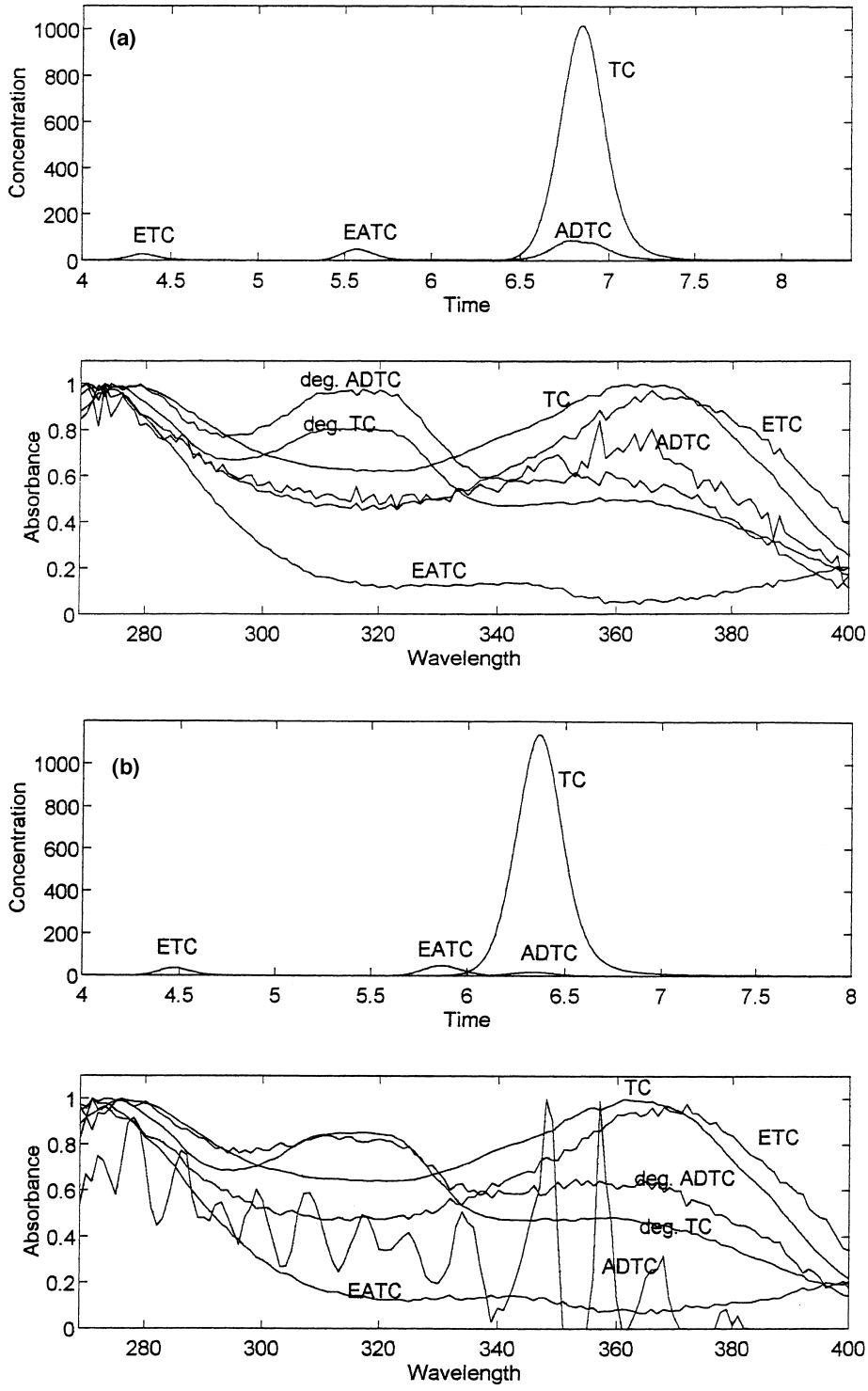


Fig. 8. Recovered concentration profiles and spectra of matrices M765 (a) and M740 (b) after application of MCR-ALS to three-way data sets formed by the sample mixture and the TC and ADTC standards.

ADTC still is not clearly resolved because of the very low concentration for ADTC in the matrix ADTC_TLC. The resolution of matrix M720 was not satisfactory. One of the components was eliminated during the MCR-ALS procedure. This can be explained by the fact that the retention times for EATC and TC are very similar at pH 7.20 and there is no additional information concerning EATC in the three-way matrix. Due to this the spectrum of EATC is very similar with the spectrum of ADTC and TC. The resolution of M700, using spectral initial estimates, resulted in a larger peak area in the concentration profile for component ADTC than for the main component TC. This can be due to the high overlap in the chromatographic and the spectral direction of both components.

The three-way analysis of the matrices was also studied using concentration initial estimates. The concentration initial estimates were built by placing the concentration profiles related to each HPLC–DAD run one on top of each other sorted as the matrices are in the three-way data set. The concentration profiles related to each matrix were obtained with MCR-ALS of the single two-way data sets using spectra selected with OPA. Selectivity was added for ETC and EATC in the matrices M765, M740 and M700 by setting the concentrations of the absent species equal to zero. The resolution results for the matrices M765 and M740 did not improve. No concentration initial estimates were used for the resolution of matrix M720 due to the high peak overlap of EATC, ADTC and TC at this pH. The three-way resolution of M700 gave the same results as the two-way resolution using spectral estimates obtained with OPA (see Fig. 6d).

The relative small fitting errors in Table 3, and the resolved profiles in Fig. 8a,b point to a general improvement in the resolution results with respect to the analysis of individual two-way matrices, but it is not as good as could be expected because the standards ADTC_TLC and TC_TLC are not pure (both have degradation products) and are in a much lower concentration than the compounds in the mixtures. The fitting errors, reported in Table 3, are obtained before divergence of the MCR-ALS optimisation process appears.

5.2.3. MCR-ALS applied to three-way data to solve the problem of small pH changes of the mobile phase in the USP XXIII method

The previous discussion and the reported ruggedness tests [17,18] indicate that small pH changes in the mobile phase of the studied USP XXIII method result in resolution changes and reversals of TC and its impurities. The application of three-way MCR-ALS was investigated in this particular case to study the gain in resolution for the matrices M750, M740, M720 and M700 by combining them with matrix M765 in a three-way data set as follows:

$$\begin{bmatrix} \text{M765} \\ \text{M750} \\ \text{M740} \\ \text{M720} \\ \text{M700} \end{bmatrix}$$

Fig. 2a–d shows that the separation of TC from its impurities is optimal at the nominal pH (7.65) in the studied pH range. The spectra selected with OPA in the matrix M765 for ETC, EATC, TC and ADTC are, therefore, used as initial estimates for the application of MCR-ALS. The spectra for ETC and EATC were fixed during the MCR-ALS optimisation

Table 3
Fitting error of the three-way resolution results with respect to the PCA reconstructed data (PCA) and the experimental data (exp)

Matrix	Fitting error before divergence appears: three-way resolution			
	Spectral initial estimates		Concentration initial estimates	
	PCA	Exp	PCA	Exp
M765	1.389	1.394	1.501	1.505
M740	2.907	2.910	3.164	3.166
M700	1.689	1.694	4.667	4.668

Each three-way data set includes the matrices of TC and ADTC standards (TC_TLC and ADTC_TLC) and one of the matrices on the left column of this table.

because both components were well separated from each other and from TC in the matrix M765. Convergence was obtained at iteration 11. The fitting error of the resolution results is 0.8459% (PCA) and 0.8798% (exp). The resolved spectra and concentration profiles in Fig. 9 show that the second peak maximum in the spectra for ADTC and TC is clearly located at different wavelengths. The concentration profiles indicate the expected elution order of TC and the impurities. The peak area in the concentration profiles for ADTC and TC are not in correspondence with the fact that ADTC is present as an impurity under the main component TC. From pH 7.40 on the peak area of ADTC becomes even bigger than the one of TC. The reason why the perfect resolution of ADTC is not possible is again the large overlap of both components in the chromatographic and spectral directions in all matrices. In this extreme case, the complete resolution of these two compounds could only be reached if pure standards of both analytes were combined with the mixture matrices.

The simultaneous analysis of the matrices M765, M750, M740 and M700 with MCR-ALS using concentration initial estimates did not improve the resolution results for the component ADTC. The spectral estimates are thus more informative than the concentration profiles to discriminate ADTC from TC. The concentration estimates are shown in Fig. 10. The matrix M720 cannot be included in the three-way data matrix due to the high peak overlap of EATC, ADTC and TC. For the matrices M765, M750 and M740 the concentration estimates are obtained from the simultaneous analysis of each matrix with the matrices ADTC_TLC and TC_TLC using spectral initial estimates. The concentration profiles for M700 are obtained from the three-way analysis of M700 with ADTC_TLC and TC_TLC using concentration initial estimates.

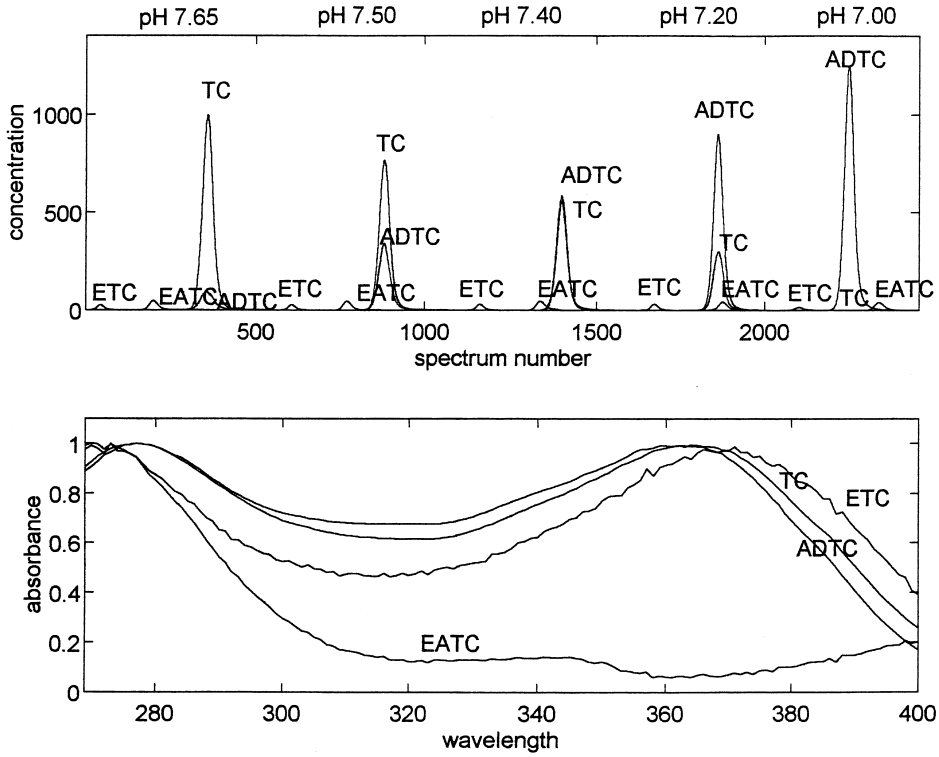


Fig. 9. Concentration profiles and spectra obtained from the simultaneous analysis of M765, M750, M740, M720 and M700 with MCR-ALS using spectral estimates obtained from M765.

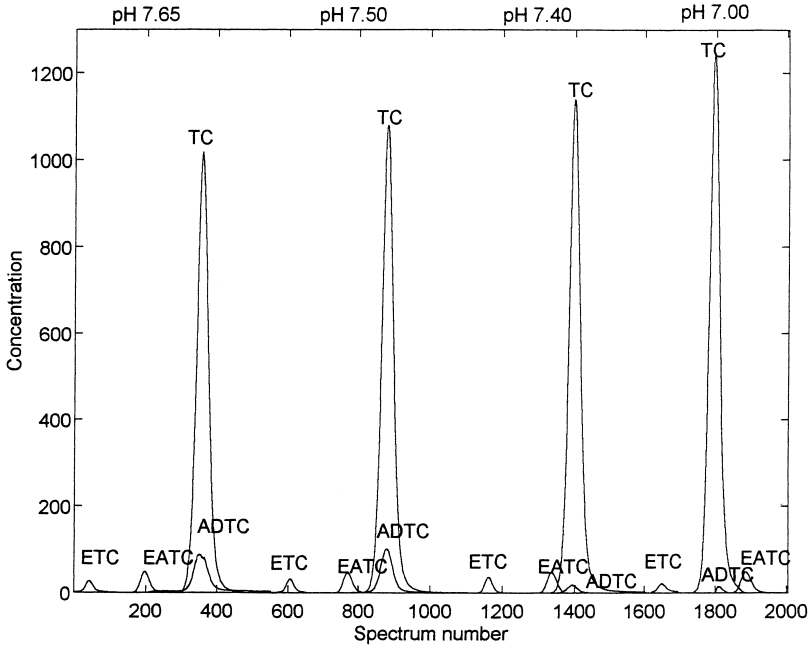


Fig. 10. Concentration initial estimates for the simultaneous analysis of M765, M750, M740 and M700.

6. Conclusion

The multivariate mixture analysis methods OPA and FSW-EFA were successfully applied for the detection of the impurity EATC (2%) under the chromatographic peak of the main component TC, even when their retention times coincide. The presence of EATC was clearly observed by simple visual inspection of the OPA and FSW-EFA plots. This impurity could not be detected with the USP XXIII univariate method. More reliable information about the purity of substances can thus be obtained by applying multivariate mixture analysis methods on data recorded with hyphenated techniques.

The use of OPA and FSW-EFA for the detection of the impurity ADTC ($<1\%$) under the peak of the major component TC is not so successful due to the fact that both components have a very similar spectrum and elute with a small chromatographic resolution. It is not an easy task to distinguish the presence of ADTC from artefacts in the measurements. The OPA plots obtained for the recorded data sets show some features, which could be interpreted as a non-linearity artefact. Nevertheless, the presence of ADTC under TC was confirmed by TLC and by the application of OPA on the data sets recorded with HPLC–DAD for the ADTC and TC spots observed on the TLC plate. Due to this the features observed in the OPA plots under the peak of TC were selected for the impurity ADTC. Spectra were selected with OPA until the calculated dissimilarities became very small and represent only noise.

Once the presence of impurities has been detected, their concentration profiles and spectra can be modelled by using the MCR-ALS resolution method. The ambiguities present in the results coming from the resolution of the individual matrices are considerably diminished when several matrices are treated together. In this sense, including matrices of TC and ADTC standards in the analysis of each individual mixture matrix led to a general improvement in the results, though not as important as could be expected because of the low concentration of the standards used and the presence of TC and ADTC degradation products in these reference matrices. Much more benefit was obtained doing the simultaneous analysis of multiple chromatographic runs obtained at different pH values. In this case, the better chromato-

graphic separation of certain matrices had positive effects on the resolution results of other chromatographic runs with a higher overlap in the chromatographic direction. The three-way analysis of the different chromatographic runs assumes that there is only one pure spectrum per compound present in the studied pH range.

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