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Improved LC of minocycline drug substance

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Abstract

An isocratic liquid chromatographic method is described for the separation of minocycline and its impurities. This method uses XTerra RP-18, 5 μm (25 cm × 4.6 mm I.D.), a silica-based stationary phase with reduced silanol activity. A mobile phase composed of acetonitrile–0.2 M tetrabutylammonium hydrogen sulphate pH 6.5–0.2 M ethylenediaminetetraacetic acid pH 6.5–water (20:20:20:40; v/v/v/v) was used at a flow rate of 1 ml/min. The column temperature was set at 35 °C. UV detection was performed at 280 nm. Optimisation of the separation method and a robustness study were performed by means of a central composite experimental design. The method allows to separate minocycline from known impurities. Some unidentified impurities are also separated. The total time of analysis is less than 20 min.

Keywords: Liquid chromatography; Minocycline; Purity testing

1. Introduction

Minocycline (MC) is a broad-spectrum antibiotic used in human medicine and also as animal feed supplement [1,2]. Since it is produced by chemical modification of demeclocycline, it can contain several structurally related compounds such as 6-deoxy-6-demethyltetracycline (6-DODMTC), 9-minocycline (9-MC), 7-didemethylminocycline (7-DDMMC), 7-monodemethylminocycline (7-MDMMC). As in the case of other tetracyclines not carrying a 5-hydroxyl group, 4-epimerization is favoured in slightly acidic to neutral solutions and leads to the formation of 4-epiminocycline (4-EMC), a potential degradation product and the main impurity [1]. The structure of MC and its most common impurities are shown in Fig. 1.

Similar LC methods for the analysis of MC are prescribed by the United States Pharmacopeia (USP) [3] and the European Pharmacopoeia (Ph. Eur.) [4]. An octylsilyl silica gel stationary phase is used. The performance of these methods has been discussed elsewhere [5].

An improved LC method for MC and its impurities, using a polymer column with alkaline mobile phase was described by Naidong et al. [6]. Another method using also a polymeric column, but with an acidic mobile phase was described by Bryan and Stewart [7]. Although these methods use columns that have a high stability, the peak shape is rather poor. Some LC and LC–MS methods were reported for the determination of MC in plasma [8–11]. LC determination of MC with electrochemical detection was reported [12]. The method was used to separate MC from other tetracycline antibiotics, but not for purity control of MC. Capillary zone electrophoresis (CZE) and thin-layer chromatography were also used for the analysis of MC [13,14].

Waters XTerra is a relatively recent addition to the large number of LC reversed phase columns based on silica gel. It contains methyl groups that are directly attached to silicon atoms in order to reduce the number of free silanol groups. Additionally, the organosilane contains a polar carbamate group, which shields the free surface silanol groups from interaction with basic compounds during chromatography. This stationary phase was already used with success for the analysis of tetracycline (TC), oxytetracycline (OTC) and doxycycline (DOX) [15–17].

The objective of this work was to develop an improved LC method for the analysis of MC using XTerra RP-18 as a stationary phase.

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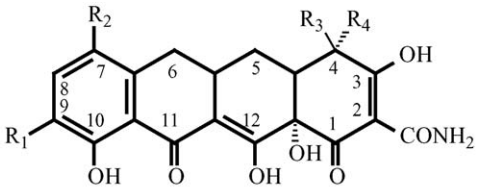

	R_1	R_2	R_3	R_4
Minocycline (MC)	H	$N(CH_3)_2$	H	$N(CH_3)_2$
6-Deoxy-6-demethyl-tetracycline (6-DODMTC)	H	H	H	$N(CH_3)_2$
7-Didemethylminocycline (7-DDMMC)	H	NH_2	H	$N(CH_3)_2$
7- Monodemethylminocycline (7-MDMMC)	H	$NHCH_3$	H	$N(CH_3)_2$
9-Minocycline (9-MC)	$N(CH_3)_2$	H	H	$N(CH_3)_2$
4-Epiminocycline (4-EMC)	H	$N(CH_3)_2$	$N(CH_3)_2$	H

Fig. 1. Chemical structures of MC and related substances.

2. Experimental

2.1. Reagents and samples

Acetonitrile HPLC grade, tetrabutylammonium hydrogen sulphate (TBA) and ethylenediaminetetraacetic acid (EDTA) were purchased from Acros Organics (Geel, Belgium). Concentrated ammonia was obtained from BDH (Pool, England). Water was purified in the laboratory by distillation of demineralised water. Commercial MC samples were from Lederle laboratories (Louvain-la-Neuve, Belgium), and Certa (Braine l′Alleud, Belgium). 9-MC, 4-EMC, 6-DODMTC and 7-DDMMC were obtained from Lederle laboratories. 7-MDMMC was prepared in the laboratory by methylation of 7-DDMMC and isolated by a thin-layer chromatographic method previously described for identification of tetracyclines [18]. Demethylchlortetracycline (DMCTC) is available from the Ph. Eur. (Strasbourg, France). All the reference substances were hydrochloride salts. MC samples were prepared at a concentration of 0.5 mg/ml. Impurities were prepared at a final concentration of 0.025 mg/ml. Ten percent EDTA solution pH 6.5 was used as a solvent for the samples and the impurities.

2.2. Instrumentation and liquid chromatographic conditions

The LC apparatus consisted of a P680 LC pump (Dionex, Sunnyvale, CA USA), a UVD 170U UV–vis detector (Dionex) and an ASI-100 Series autosampler (Dionex).

The XTerra RP-18 column (25 cm × 4.6 mm I.D.), 5 μm (Waters, Milford, Massachusetts, USA) was kept at 35 °C in a water bath heated by means of a Julabo ED thermostat (Julabo, Seelbach, Germany). The flow rate was 1 ml/min. The injection volume was 20 μl.

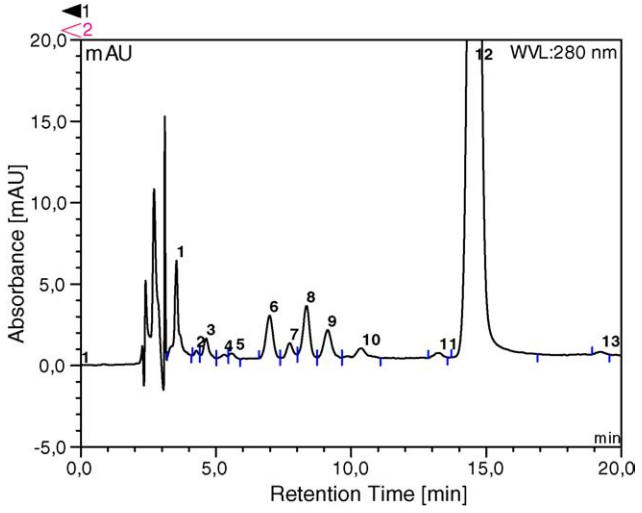

Fig. 2. A typical chromatogram of a commercial MC sample (0.5 mg/ml) spiked with 7-MDMMC, 9-MC and 7-DDMMC: (1) unknown 1, (2) unknown 2, (3) 7-MDMMC, (4) unknown 3, (5) unknown 4 (6) 7-DDMMC, (7) unknown 5, (8) 4-EMC, (9) 9-MC, (10) 6-DODMTC, (11) unknown 6, (12) MC and (13) unknown 7. Column: XTerra RP-18, 5 μm, 250 mm × 4.6 mm I.D., maintained at 35 °C. Mobile phase: CH_3CN–0.2 M TBA pH 6.5–0.2 M EDTA pH 6.5–water (20:20:20:40; v/v/v/v), detection: UV at 280 nm; flow rate: 1.0 ml/min; injection volume: 20 μl.

2.3. Mobile phase

The mobile phase consisted of acetonitrile–0.2 M tetrabutylammonium hydrogen sulphate pH 6.5–0.2 M ethylenediaminetetraacetic acid pH 6.5–water (20:20:20:40; v/v/v/v). EDTA and TBA solutions were adjusted to the required pH with concentrated ammonia before bringing up to volume.

2.4. Experimental design

A robustness study was performed by means of an experimental design and multivariate analysis using Modde 5.0 software (Umetrics, Umea, Sweden). A central composite design was applied. The central composite design is composed of a full or fractional factorial design, star points and a replicated centre point. The star points enable the model to estimate the curvature response. These star points are located at the centre and both extreme levels of the experimental domain. For a normal central composite design, which includes the points of a two level full factorial design, the number of runs is equal to $2^k + 2k + n$. k is the number of parameters and n is the number of centre points. In this study, five parameters (percentage of acetonitrile, percentage of TBA solution, percentage of EDTA solution, column temperature and mobile phase pH) were investigated. With this number

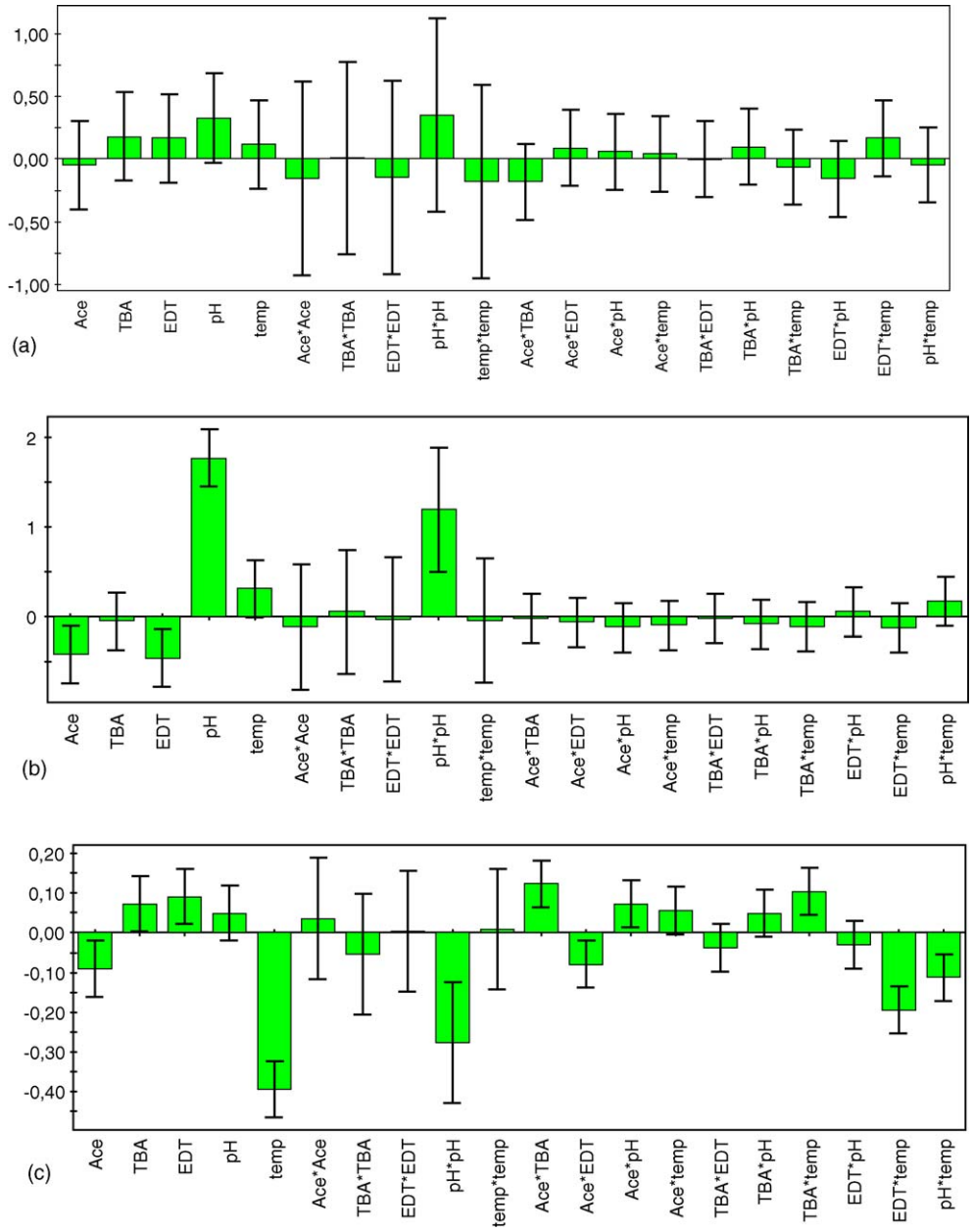

Fig. 3. Regression coefficient plots for the separation of the pairs: (a) unknown 2–4-EMC (Rs_1), (b) EMC–9-MC (Rs_2) and (c) 9-MC–6-DODMTC (Rs_3). Ace = percentage of acetonitrile in the mobile phase; TBA = percentage of 0.2 M TBA solution in the mobile phase; EDT = percentage of 0.2 M EDTA solution in the mobile phase; pH = mobile phase pH; temp = column temperature.

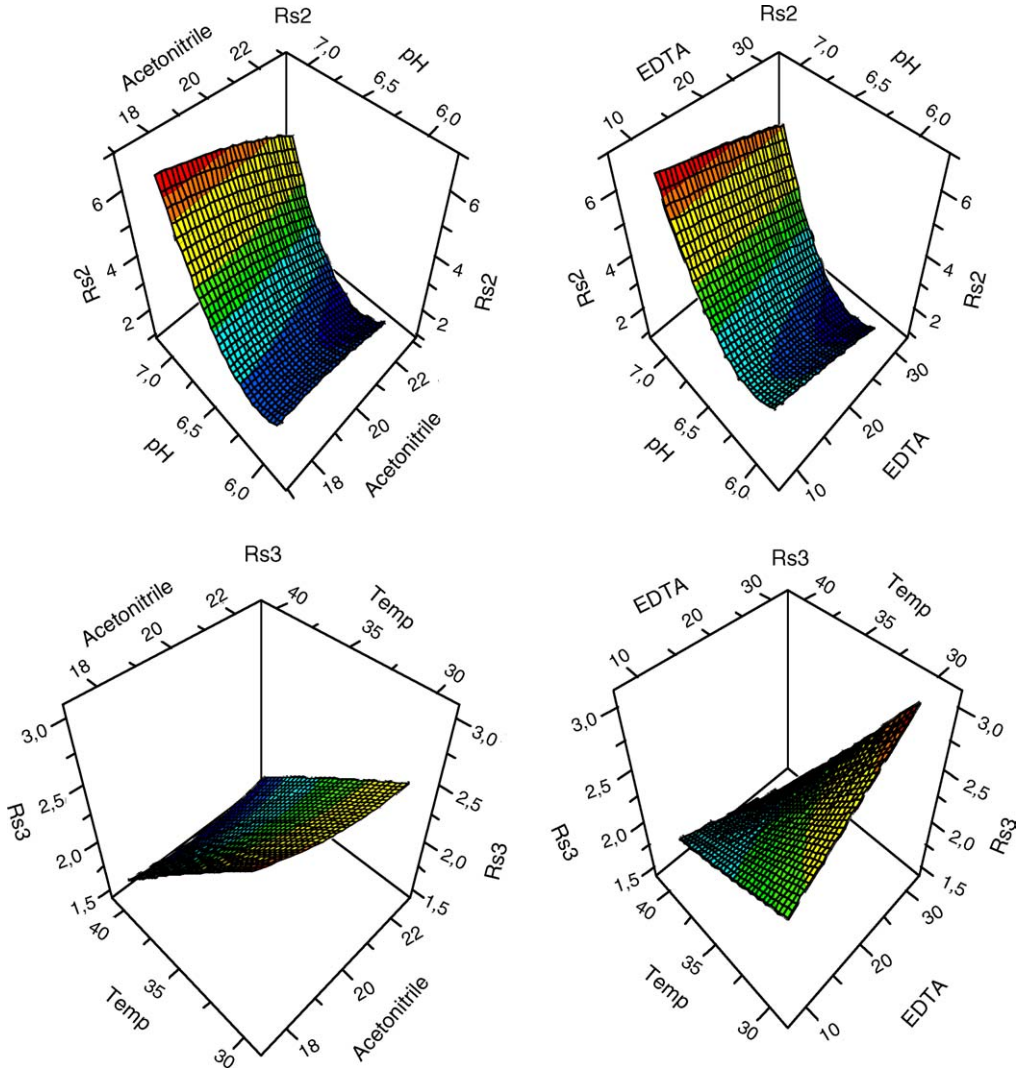

Fig. 4. Response surface plots for the separation of the pairs EMC–9-MC (Rs_2) and 9-MC–6-DODMTC (Rs_3).

of parameters and 3 centre points, a normal central composite design would result in a number of runs equal to 45. In order to reduce the number of runs, a central composite design which includes the points of a two level half fractional factorial design was chosen, with a number of runs equal to $2^{k-1} + 2k + n = 29$. The statistical relationship between a response Y and the experimental variables X_i, X_j, ... is of the following form:

$$Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ij} X_i X_j + \beta_{ii} X_i^2 + \beta_{jj} X_j^2 + \cdots + E$$

where the β's are the regression coefficients and E is the overall experimental error.

The linear coefficients for the experimental variables, β_i and β_j describe their quantitative effect in the model. The cross coefficient β_{ij} measures the interaction effect between the variables and the square terms $\beta_{ii} X_i^2$ and $\beta_{jj} X_j^2$ describe non linear effects on the response.

3. Results and discussion

3.1. Method development

The development of the method was based on the experience obtained with the methods previously developed for the analysis of TC, DOX and OTC [14–16]. These methods use XTerra

Table 1
Chromatographic parameter settings applied in the central composite design, corresponding to low (−), central (0) and high (+) levels

Chromatographic parameter	Low value (−)	Central value (0)	High value (+)
Percentage of acetonitrile	17.5	20	22.5
Percentage of 0.2 M TBA solution	10	20	30
Percentage of 0.2 M EDTA solution	10	20	30
pH	6	6.5	7
Temperature (°C)	30	35	40

Table 2
Repeatability data for the analysis of MC and some of its impurities

	Unknown 5	4-EMC	9-MC	6-DODMTC	Unknown 3	MC	No. of experiments
Component level (%)	0.6	0.9	1.5	0.8	0.3	96.0	
Intra-day R.S.D. (%)							
Day 1	1.5	2.8	1.0	5.7	1.2	0.2	6
Day 2	1.6	4.9	0.4	6.8	0.4	0.0	6
Inter-day R.S.D. (%)	0.6	2.3	3.0	3.6	4.5	0.9	12

RP-18 as stationary phase and mobile phases containing acetonitrile, TBA and EDTA. The mobile phase pH, the column temperature and the amounts of acetonitrile, TBA and EDTA were investigated in order to find the best conditions giving a good separation of MC and its impurities. The pH of EDTA and TBA solutions was investigated in the range from 6 to 7.5. A pH of 6.5 was chosen as a compromise between good separation and column stability. The percentage of the EDTA solution was investigated in the range from 10 to 30%. EDTA captures the metal ion impurities in the mobile phase through complexation and thus, prevents their interaction with the tetracyclines. The EDTA solution is a buffer as well and upon increasing its amount, the symmetry was improved. However, increasing EDTA also resulted in a decrease of the sensitivity. So, an amount of 20% was chosen as a compromise. To be able to use high concentration of EDTA, an in situ formed ammonium salt, which is more soluble than the sodium salt, was used. TBA as an ion pairing agent interacts with negatively charged analytes, thus forming neutral pairs, which in turn interact better with the stationary phase leading to an improved selectivity. It also improves peak symmetry since it competes with analyte ions for the residual silanol groups, thereby reducing peak tailing. The amount of TBA solution was investigated in the range 10–30%. Twenty percent of TBA was chosen as it allowed to achieve sufficient resolution and good peak shape. The acetonitrile content was investigated in the range 17.5–22.5%. A concentration of 20% was chosen as a compromise in order to achieve a good resolution for all peaks and a short analysis time. The column temperature was investigated between 30 and 40 °C. 35 °C was chosen in order to achieve good separation while preserving column stability.

A typical chromatogram obtained by analysing a spiked commercial MC sample using the chosen chromatographic conditions is shown in Fig. 2. It is observed that MC is well separated from its known impurities. Some impurities of unknown identity are also separated. DMCTC, which is used as the starting material for the semi-synthesis of MC, was not found in the sample analysed. Although the analysis of this compound indicated that it is coeluted with the compound unknown 5, further investigation by mass spectrometry (MS) revealed a difference in identity between the two compounds. Exploring the separation method using an experimental design (described below) indicated that the method was optimal in the ranges examined. Compared to a previously published LC method, using a polymeric stationary phase [6], the method described here gives better efficiency and shorter analysis time. The main peak is better separated from surrounding impurities, which allows better quantitation. Compared to a CZE method [13], the proposed method shows higher selectivity and similar analysis time.

Table 3
Limits of quantification and the corresponding R.S.D. values for MC and some of its impurities

	MC	4-EMC	6-DODMTC
LOQ			
Percent	0.025	0.025	0.025
Mass on column (ng)	2.5	2.5	2.5
R.S.D. ($n = 6$)	1.4	10.2	4.2

3.2. Robustness study

The robustness study was performed by means of an experimental design as reported under 2.4. The different chromatographic parameter settings in the design are given in Table 1 and their individual and interaction effects on the resolution for the most important separations, namely unknown 5 and 4-EMC (Rs_1), 4-EMC and 9-MC (Rs_2) and 9-MC and 6-DODMTC (Rs_3), are summarised in Fig. 3. The plots consist of bars, which correspond to the regression coefficients. The magnitude of variable effects is proportional to the regression coefficients. The 95% confidence limits are expressed by using error lines. A regression coefficient smaller than the error line interval shows that the variation of the response caused by changing the variable is smaller than the experimental error. Therefore, in this case the effect of variable change would be considered insignificant when compared to the response.

Table 4
Linearity data of MC and some of its impurities

	Concentration range (%)	Regression equation	$S_{y,x}$	R^2	n_c	n_i
MC	25–125	$y = 2.51x - 0.47$	3.5086	0.999	5	3
6-DODMTC	0.025–5	$y = 3.41x - 0.10$	0.1251	0.999	5	3
4-EMC	0.025–5	$y = 1.97x + 0.01$	0.0435	0.999	5	3

R^2 = coefficient of determination; $S_{y,x}$ = standard error of estimate; n_c = number of experimental concentrations studied; n_i = number of injections for each concentration; y = peak area; x = concentration (%).

Table 5
Composition of commercial drug substances

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
Unknown 1	ND	ND	0.1 (14.3)	ND	ND	ND	ND	ND
Unknown 2	ND	ND	0.1 (13.3)	ND	ND	ND	ND	ND
7-MDMMC	< LOQ	0.1 (6.9)	0.1 (9.1)	ND	ND	ND	<LOQ	ND
Unknown 3	ND	ND	0.1 (10.8)	ND	ND	ND	ND	ND
Unknown 4	< LOQ	0.1 (10.2)	0.2 (6.3)	< LOQ	< LOQ	< LOQ	0.1 (15.7)	< LOQ
7-DDMMC	ND	< LOQ	0.1 (21.7)	ND	ND	ND	ND	ND
Unknown 5	0.3 (1.9)	0.4 (1.4)	0.6 (1.9)	0.1 (7.9)	0.1 (12.5)	0.3 (0.1)	0.1 (8.2)	0.7 (0.8)
4-EMC	0.4 (1.5)	0.6 (1.0)	0.6 (1.7)	0.4 (1.7)	0.4 (4.4)	0.5 (1.1)	0.8 (2.6)	0.2 (12.9)
9-MC	ND	< LOQ	<LOQ	<LOQ	ND	ND	ND	ND
6-DODMTC	0.3 (2.1)	0.3 (1.7)	0.8 (2.6)	0.1 (17.3)	0.1 (17.3)	0.2 (2.4)	0.5 (19.3)	< LOQ
Unknown 6	0.2 (3.5)	0.2 (3.5)	0.3 (1.7)	ND	<LOQ	0.2 (3.5)	0.2 (5.6)	0.1 (5.6)
MC	98.7 (0.1)	98.2 (0.1)	96.7 (0.1)	99.4 (0.1)	99.4 (0.1)	98.5 (0.1)	98.1 (0.1)	98.9 (0.1)
Unknown 7	0.1 (5.1)	0.2 (2.8)	0.5 (2.4)	0.1 (8.4)	0.1 (4.6)	0.2 (2.7)	0.2 (4.7)	<LOQ

ND = not detected. The R.S.D. ($n = 3$) are given in parentheses.

In the range investigated, none of the parameters has a significant effect on Rs_1. It is observed that the pH has the most important effect on Rs_2, followed by EDTA and acetonitrile. The effect is positive for the pH, while it is negative for EDTA and acetonitrile. This means that an increase of the pH improves the separation, but an increase of the amounts of acetonitrile or of EDTA decreases the separation. All interaction effects are found to be not significant. The temperature has the most important effect on Rs_3, followed by EDTA and acetonitrile. This effect is negative for the temperature and acetonitrile while it is positive for EDTA. For the pH, a quadratic effect is observed, while the linear coefficient is not significant. TBA does not have a significant effect. No important interaction effects are noticed. In order to estimate better the influence of the most important parameters on Rs_2 and Rs_3, response surface plots were constructed. Fig. 4 shows, in the top row, the variation of Rs_2 as a function of pH, EDTA and acetonitrile, the other parameters being kept constant at their central values. The lower row shows the variation of Rs_3 as a function of temperature, EDTA and acetonitrile while the other parameters are kept constant at their central values. It is observed that in the ranges examined, Rs_2 and Rs_3 are always well above 1.5. This means that the chosen chromatographic conditions are a good compromise for optimal separation and that small variations in these conditions will not have a harmful effect on the separation of the different pairs investigated. It can therefore be concluded that the developed method is robust.

3.3. Quantitative aspects

The repeatability of the method was assessed by analysing six times a 0.5 mg/ml solution of a commercial MC sample. The levels of the different components studied and the relative standard deviation (R.S.D.) of the peak areas for both intra- and inter-day repeatability are summarised in Table 2. The results obtained demonstrate the good precision of the method. The limits of quantitation (corresponding to a signal-to-noise ratio of 10) and the corresponding R.S.D. values obtained for MC and some of its impurities are summarized in Table 3. Comparing these data to those described for a CZE method [13], the LC method developed here shows a higher sensitivity. This method shows a similar sensitivity but higher efficiency and better peak shape compared to the previously published LC method using a polymer column [6].

The linearity was checked by separate analyses of solutions of MC and its available impurities. The concentrations examined were in the range 25–125% for MC and in the range LOQ–5% for 4-EMC and 6-DODMTC. The percentages were calculated versus the amount of MC injected on the column in an analysis as described in Section 2 (10 μg = 100%). The linearity data obtained for MC and its impurities are summarised in Table 4. The results obtained demonstrate the good linearity of the method. The proposed method was applied for the analysis of commercial samples. The composition was calculated by normalization, using all peak areas above the LOQ. Data obtained are summarized in Table 5. It is observed that the chromatographic purity of the samples analyzed varies from 96.7 to 99.4%.

4. Conclusion

An improved LC method was developed for the analysis of MC. This method allows complete separation of MC from eight components of which five are known MC impurities and three are unidentified peaks found in several commercial samples. The developed method shows good sensitivity, linearity and proved to be robust. All components are eluted in less than 20 min.

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