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Development and validation of an improved method for the analysis of vancomycin by liquid chromatography Selectivity of reversed-phase columns towards vancomycin components

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Abstract

The current method prescribed in official monographs for the purity control of vancomycin is inappropriate in that several components are not separated from each other and other components are coeluted with the main component vancomycin B. The method uses an ODS column at pH 3.2. In this study, several changes were introduced in order to improve the separation. The optimization of the separation method at low pH indicated that pH 1.7 was optimum and that the use of dioxane as organic modifier drastically improved the separation. These conditions were used to test a set of more than 40 reversed-phase columns for their selectivity towards vancomycin components. The selection of the most suitable columns was performed by means of principal component analysis. Most of these columns did not allow the separation of didechlorovancomycin from monodechlorovancomycin 1. It was found that neutral to slightly alkaline mobile phases allowed better separation. Further optimization of the separation method and a robustness study were performed by means of experimental design. This optimization indicated that pH 7.7 was optimum and gradient elution was also used to effect complete analysis. The final method uses a Kromasil column and the mobile phase comprises dioxane, water and ammonium formate solution pH 7.7. The separation of monodechlorovancomycin 2 and of some unknown impurities from the main component vancomycin B is described for the first time. The method shows good repeatability, linearity and sensitivity.

Keywords: Validation; Stationary phases, LC; Pharmaceutical analysis; Vancomycin; Antibiotics; Glycopeptides; Peptides

1. Introduction

Vancomycin (VM) is a glycopeptide antibiotic used for the treatment of severe infections caused by Gram-positive bacteria, including methicillin-resistant and oxacillin-resistant staphylococci [1–7]. It is also the treatment of choice of bacterial infections in patients allergic to β-lactam antibiotics [1–4,8]. VM is produced through fermentation by *Amycolatopsis orientalis* and it appears as a mixture of similarly structured compounds of which many components

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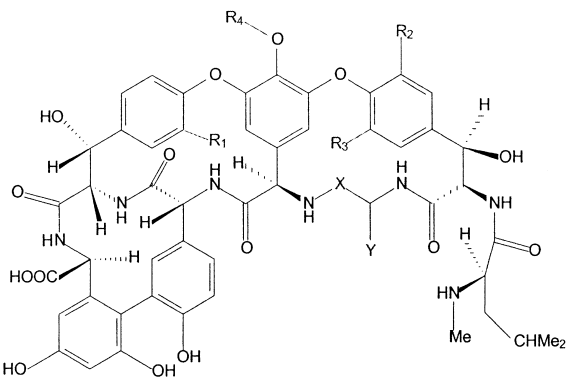
remain to be identified [9–12]. Monodechlorovancomycin 2 (MDCV 2) is a side-product isolated from fermentation broth. VM can be selectively dehalogenated to give monodechlorovancomycin 1 (MDCV 1); further reduction gives didechlorovancomycin (DDCV) [13]. VM degrades into a crystalline degradation product (CDP-I) by hydrolytic loss of ammonia [14,15]. CDP-I exists in two isomeric forms, the major form (CDPM) and the minor form (CDPm) [15–18]. The conversion of VM to CDP-I passes through an intermediate, the CDP-I intermediate (CDPinter). Aglucovancomycin (AGLUV) and desvancosaminylvancomycin (DESV) are other degradation products resulting from the loss of the disaccharide moiety and the vancosamine sugar, respectively [19]. The structures of VM and the above mentioned impurities are shown in Fig. 1. The potential impurities are therapeutically less active than the parent molecule vancomycin B (VM B) [20]. The two isomers of CDP-I (CDPM and CDPm) are even therapeutically inactive [14,15,20]. It is therefore important to have available selective and



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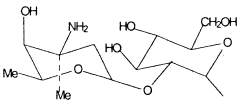
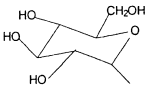
A 

B 

C H

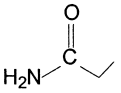
X	Y	R_1	R_2	R_3	R_4	Compound
		Cl	Cl	H	A	VM B
		Cl	H	H	A	MDCV 1
		H	Cl	H	A	MDCV 2
		H	H	H	A	DDCV
		Cl	Cl	H	B	DESV
		Cl	Cl	H	C	AGLUV
		Cl	H	Cl	A	CDPM
		Cl	Cl	H	A	CDPm

Fig. 1. Structures of vancomycin B and related substances.

sensitive analytical methods that are able to separate these impurities and to allow a better understanding of the composition of VM.

Several methods have been used for the analysis of VM, such as thin-layer chromatography [21], microbiological assay [22,23], fluorescence polarization immunoassay [1,23–27], radioimmunoassay [23,28], enzyme multiplied immunoassay [29], capillary electrophoresis (CE) [30,31] and liquid chromatography (LC) [12,32–51]. Most of these methods were used to monitor the VM concentration in blood. The method described by Kang et al. [31] allows the purity control of VM. However, the technique used was micellar electrokinetic capillary chromatography (MECC). Only a few LC methods were used for the purity control of VM [12,41,50]. The LC method described by Inman [12] was the basis for the current official method in the European Pharmacopoeia (Ph. Eur.) [52] and in the United States Pharmacopeia (USP) [53]. This method is inappropriate in that several components are not separated and there are compounds that are coeluted with the main component VM B. Except for MDCV 2, the nature and the biological effects of the potentially interfering compounds are not known and improved separation methods are needed.

In this work, we developed a more selective and sensitive LC method for the analysis of VM. The use of a volatile salt in the mobile phase should allow this method to be compatible with mass spectrometry (MS). The selectivity of a set of more than 40 reversed-phase columns towards VM components was also investigated.

2. Experimental

2.1. Reagents and samples

HPLC-grade acetonitrile was purchased from Biosolve (Valkenswaard, The Netherlands). HPLC-grade dioxane, 2-methoxyethanol, methyl acetate and formic acid were purchased from Acros Organics (Geel, Belgium). Concentrated ammonia, tetrahydrofuran and 2-propanol were from Riedel-de Haën (Seelze, Germany). HPLC-grade methanol was purchased from Fisher Chemicals (Loughborough, UK). 2-Methyl-2-propanol (Merck Eurolab, Leuven, Belgium) was distilled before use. Water was purified in the laboratory by distillation of demineralised water. Reference substances of VM B, MDCV 1, MDCV 2, DDCV, DESV, AGLUV, CDPinter, CDPM and CDPm were obtained from Abbott Labs. (Abbott Park, IL, USA). Vancomycin samples were prepared at a concentration of about 2 mg/ml. Solutions of reference substances were prepared at a final concentration of about 50 μg/ml. For the assay, a 2 mg/ml solution of VM B reference substance was prepared. The samples and the reference substances were dissolved in water for the experiments performed at low pH. In the final method, they were dissolved in the mobile phase. Sample solutions slightly degraded within 1 day when they were kept at room temperature. However these sample solutions could be used for several days when they were stored at 4 °C.

2.2. Instrumentation and liquid chromatographic conditions

The LC apparatus (equipment I) consisted of an L-6200 Intelligent Pump (Merck–Hitachi, Darmstadt, Germany), an autosampler Model 655A-40 (Merck–Hitachi), a linear UVIS 200 UV detector [Thermo Separation Products (TSP), San Jose, CA, USA] set at 280 nm and an integrator Model HP 3396 Series III (Hewlett-Packard, Avondale, PA, USA). The column was kept in a water bath at 35 °C. A Kromasil C_{18} column (250×4.6 mm I.D.), 5 μm (Akzo Nobel, Bohus, Sweden) was used. The flow-rate was 1.0 ml/min. The injection volume was 20 μl.

For the intermediate precision study, analyses were carried out using a new Kromasil C_{18} column (250×4.6 mm I.D.), 5 μm from a different batch and different LC equipment (equipment II) consisting of an L-6200 Intelligent Pump (Merck–Hitachi), a TSP Spectra Series AS 100 autosampler set to inject 20 μl (San Jose, CA, USA), a Merck–Hitachi L-4200 UV–Vis detector set at 280 nm and ChromPerfect 4.4.23 software (Justice Laboratory Software, Fife, UK) for data acquisition.

2.3. Mobile phase

The mobile phases consisted of dioxane–0.3 *M*

ammonium formate, pH 7.7–water (A) (7:15:78, v/v) and (B) (30:15:55, v/v). To prepare the 0.3 *M* ammonium formate solution, the appropriate amount of concentrated ammonia was diluted in water and adjusted to the required pH using 10% formic acid, before bringing to volume. The mobile phases were degassed by sparging helium.

2.4. Software

Experimental design, optimization of the method and robustness evaluation were performed using Modde 4.0 statistical graphic software (Umetrics, Umea, Sweden).

Data reduction was achieved by principal component analysis (PCA) using Statistica 6.0 statistical graphic software (StatSoft, Tulsa, USA).

3. Results and discussion

3.1. Method development

The method development was started with the LC method prescribed by the Ph. Eur. and the USP for the analysis of VM. This method uses a C_{18} (250× 4.6 mm I.D.), 5 μm, stationary phase. UV detection is performed at 280 nm. Mobile phase containing triethylamine phosphate buffer pH 3.2, tetrahydrofuran, acetonitrile and water is used at a flow-rate of 1.0 ml/min. The separation obtained with this method, using a Hypersil BDS stationary phase, was not good. MDCV 1 was coeluted with DDCV and MDCV 2 was coeluted with the main component VM B. A gradient was needed to elute the most strongly retained AGLUV peak within acceptable time. But as critical separations were found only in the isocratic part of the chromatogram, further steps to improve these separations only included isocratic elution to save time. Gradient elution was added again at the end of the development, to complete the method.

Modifications of the buffer used were made in order to improve the separation and especially to make the method compatible with MS. Different salts were checked: potassium phosphate, ammonium phosphate, ammonium citrate, ammonium acetate, and ammonium formate. Ammonium formate gave the best separation and it is compatible with MS. However, the separation of MDCV 2 and some other impurities was not achieved.

The nature and the content of the organic modifier were further investigated to improve the separation. Several organic modifiers were examined: acetonitrile, tetrahydrofuran, methanol, 2-propanol, 2-methyl-2-propanol, 2-methoxyethanol, methyl acetate and dioxane. The use of dioxane drastically improved the separation. A decrease of the mobile phase pH from 3.2 to 1.7 also improved the separation. The separation of MDCV 2 from VM B was obtained when a mobile phase containing dioxane (6%) and 0.3 M ammonium formate solution, pH 1.7 (5%) was used. However, the separation of the pair DDCV–MDCV 1 was not achieved and some unknown components were eluted at the tail of the main component VM B.

3.2. Selectivity of reversed-phase columns towards VM components

The chromatographic conditions thus selected were used to test different C_{18} reversed-phase columns for their selectivity towards VM components. Specifications of the columns examined are given in Table 1. A mixture containing VM B, MDCV 1, MDCV 2, DDCV, CDPM and CDPm was analyzed in order to identify the best stationary phases giving less peak-tailing, better selectivity and efficiency. This allows one to also check for the selectivity towards the separated pair (MDCV 2–VM B) and an unseparated pair (DDCV–MDCV 1). In addition, a solution of VM commercial sample was examined in order to assess the separation of the other impurities of unknown identity (UNK). The content of dioxane in the mobile phase was adjusted to obtain similar retention times for the different columns. The column dead volume was determined by injecting a 0.01 mg/ml aqueous solution of uracil.

The influence of the different stationary phases on the separation parameters was assessed. The separation parameters monitored were the symmetry factor (SF) and the number of theoretical plates (N) for each component of the mixture analyzed as well as the selectivity (S) between closely eluted components. The theoretical plates measured for the 15 cm columns were normalized to 25 cm.

Table 1
Specifications for the examined columns

Column No.	Column name	Length (mm)	Particle size (μm)	Manufacturer/supplier
1	Hypersil BDS 5	250	5	ThermoQuest
2	Hypersil ODS 5	250	5	ThermoQuest
3	Hypersil BDS 5	250	5	ThermoQuest
4	Nucleosil 5	250	5	Macherey–Nagel/Filter Service
5	Kromasil (MN)	250	5	Macherey–Nagel/Filter Service
6	Nucleosil HD	250	5	Macherey–Nagel/Filter Service
7	Nucleosil Nautilus	250	5	Macherey–Nagel/Filter Service
8	Symmetry 5	250	5	Waters
9	Spherisorb ODS2	250	5	Waters
10	μBondapak	250	10	Waters
11	Kromasil (EKA)	250	5	Akzo Nobel/SerCoLab
12	OmniSpher	250	5	Varian
13	Supelcosil LC-18	250	5	Supelco
14	Supelcosil LC-18 DB 5	250	5	Supelco
15	Zorbax Extend C_{18}	250	5	Agilent Technologies
16	Zorbax Eclipse XDB-C_{18}	250	5	Agilent Technologies
17	Zorbax SB-C_{18}	250	5	Agilent Technologies
18	Apex ODS II	250	5	Jones Chromatography/Sopachem
19	Wakosil C_{18}HG 5–25	250	5	SGE/Achrom
20	ACE 5 C_{18}	250	5	Advanced Chrom. Tech./Achrom
21	Discovery C_{18}	250	5	Supelco
22	TracerExcel ODS A-5	250	5	Teknokroma/SerCoLab
23	Alltima C_{18} 5	250	5	Alltech
24	Brava BDS 5	250	5	Alltech
25	Platinum C_{18} 5	250	5	Alltech
26	Platinum EPS C_{18} 5	250	5	Alltech
27	LiChrospher	250	5	Merck
28	Superspher	250	5	Merck
29	Uptisphere 5 ODB	250	5	Interchrom/Achrom
30	Purospher endcapped	250	5	Merck
31	Uptisphere 5 HDOC_{18}	250	5	Interchrom/Achrom
32	Purospher STAR	250	5	Merck
33	Validated C_{18}	250	5	Perkin-Elmer
34	Spheri-5	250	5	Perkin-Elmer
35	Validated C_{18}	250	5	Perkin-Elmer
36	Luna	150	5	Phenomenex/Bester
37	Synergi	150	4	Phenomenex/Bester
38	YMC-Pack Pro C_{18}-3	150	3	YMC Sep. Techn./ThermoQuest
39	Apex Basic	250	5	Jones Chromatography/Sopachem
40	Purospher	250	5	Merck
41	Supelcosil LC-ABZ	250	5	Supelco

The same elution order was observed for the different components with most of the columns examined. Most of these columns did not allow the separation of pair DDCV–MDCV 1. However, separation of this pair could be achieved on column 39 (Apex basic) and column 40 (Purospher) and in these cases a change was observed in the elution order. A change in the elution order was also observed with column 41 (Supelcosil ABZ) but the separation of the pair DDCV–MDCV 1 was not achieved and CDPM was coeluted with MDCV 2. These columns (39, 40 and 41) were not examined further in the principal component analysis (discussed below) because the elution order was different for these columns and the overall quality of the separation obtained was poor, although the separation of the pair DDCV–MDCV 1 was achieved.

Except for the pair DDCV–MDCV 1, good selec-

tivities for the different components were observed with most of the columns of the main group (columns 1–38). In addition to the components present in the injected mixture, other impurities of unknown identity were also separated. Typical chromatograms are shown in Fig. 2.

CDPm was separated from the pair DDVC–MDCV 1 with most of the columns except Hypersil BDS (columns 1 and 3), Supelcosil LC-DB (column 14), Apex ODS (column 18) and Spheri-5 (column 34). All the columns of the main group showed very high selectivity for the pair MDCV 1–MDCV 2. A good separation of the pair VM B–MDCV 2 was achieved with most of the columns of this group except Nucleosil Nautilus (column 7), Platinum (column 25) and Platinum EPS (column 26), which allowed only an incomplete separation of that pair. The separation between CDPM and VM B was also good for most of the columns except for Nucleosil (column 4), Nucleosil Nautilus (column 7),

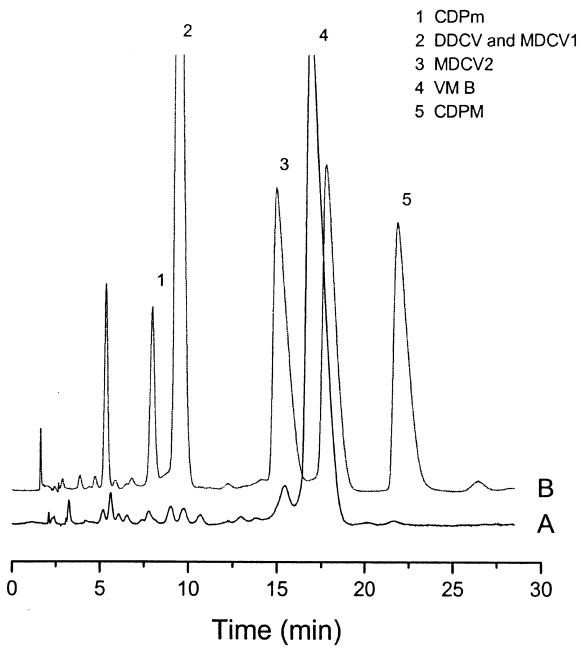


Fig. 2. Typical chromatogram of (A) VM commercial sample (2 mg/ml) and (B) a mixture of VM and some of its potential impurities (1 mg/ml) analyzed during columns investigation. Column: Symmetry (250×4.6 mm I.D.), 5 μm, maintained at 30 °C. Mobile phase: dioxane–0.3 *M* ammonium formate, pH 1.7–water (6:5:89, v/v). Detection: UV at 280 nm; flow-rate: 1.0 ml/min; injection volume: 20 μl.

μBondapak (column 10) and Platinum (column 25). The symmetry factors observed (data not shown) with the chromatographic conditions used in this study are above the limits prescribed in the European Pharmacopoeia (0.80–1.20) for almost all the components of the analysed mixture and for most of the columns of the main group. The best symmetry was observed with Validated (column 33).

The selection of the most suitable columns in the main group was achieved by PCA using Statistica 6.0 statistical software. PCA is a chemometric tool suitable for the representation of high-dimensional data in fewer dimensions. The original data set of this work is a matrix ($m \times n$) in which the objects (m) are the stationary phases and the variables (n) are the evaluated retention parameters. The content of such a large number of data can be visualized in an n-dimensional plot, which is graphically impossible. The PCA reduces the n variables to a few latent variables or principal components (PCs) without losing significant information on the original variables. These PCs can be considered as new axes drawn in the original n-dimensional space [54–56].

The first principal component (PC1) is a latent variable, which explains the largest variation in the data. The second principal component (PC2), which is orthogonal to the first one, explains the largest amount of the remaining variation. The third PC (PC3) is orthogonal to PC1 and PC2 and explains the next largest amount of the remaining variation, etc. The projections of the objects onto the PCs are called scores. A score plot represents the scores of the objects on two of the PCs, gives information about the objects and allows identifying those with closely related properties. Mathematically, a score on a PC is a weighed sum of the original variables. The weights are called loadings. Each original variable has a loading on each PC. A loading plot represents the loadings of the variables on two of the PCs. The loading plot gives information about the original variables (chromatographic parameters), i.e., about their influence and importance on the PCs. Moreover, it also gives information on the correlation of the variables [54–56].

The 12 parameters of the separation can be seen on the loading plot (see Fig. 3). The selectivity DDCV/MDCV1 is not shown in this plot as no separation was obtained with the columns. This

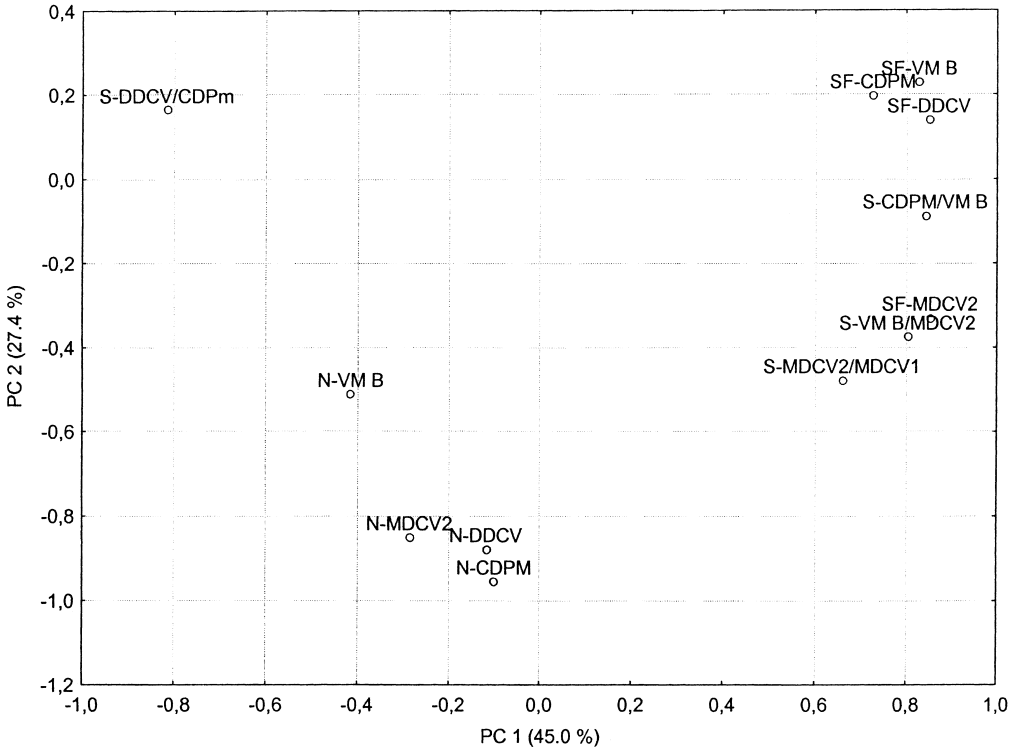


Fig. 3. Loading plot of chromatographic parameters on the first and second principal components (PC1 and PC2).

graph shows that the principal directions define the 12 variables projected along the first and second principal components (PC1 and PC2). On the left side of the graph the direction is given by the selectivity DDCV/CDPm, on the opposite side the other selectivity factors and the symmetry factors, and on the bottom the theoretical plate numbers. The score plot, which characterises the columns along the same two-dimensional plot as in the loading plot, is shown in Fig. 4.

Interpretation of both graphs indicates that the columns that have the highest efficiency (higher theoretical plates number) are projected in the bottom direction of the score plot. Those that have high symmetry factors can be found in the upper-right side of the graph. The loading of one of the selectivity factors (S-DDCV/CDPm) on the PC1 axis is negative, the others are positive; consequently columns that are situated close to zero on this axis have good selectivity for the separation of all compounds. Therefore, it can be concluded that stationary phases that are located in the bottom part of the centre of the score plot, are suitable for this separation. Appropriate columns according to the data obtained would be found inside the circle on the score plot. PCA results were confirmed by examination of the chromatograms.

3.3. Further investigation of the effect of the pH on the separation

Since none of the 40 columns investigated gave a distinctly better selectivity with mobile phase at acidic pH, it was decided to examine further the influence of pH. Among the columns showing more interesting properties, the Symmetry column was chosen for further investigations of the effect of the pH on the separation of the VM components. Different pH values from 1.7 to 8.0 were examined. No noticeable change was observed when the pH was increased up to 7. However, a definitely better separation was obtained when a pH of 8 was used.

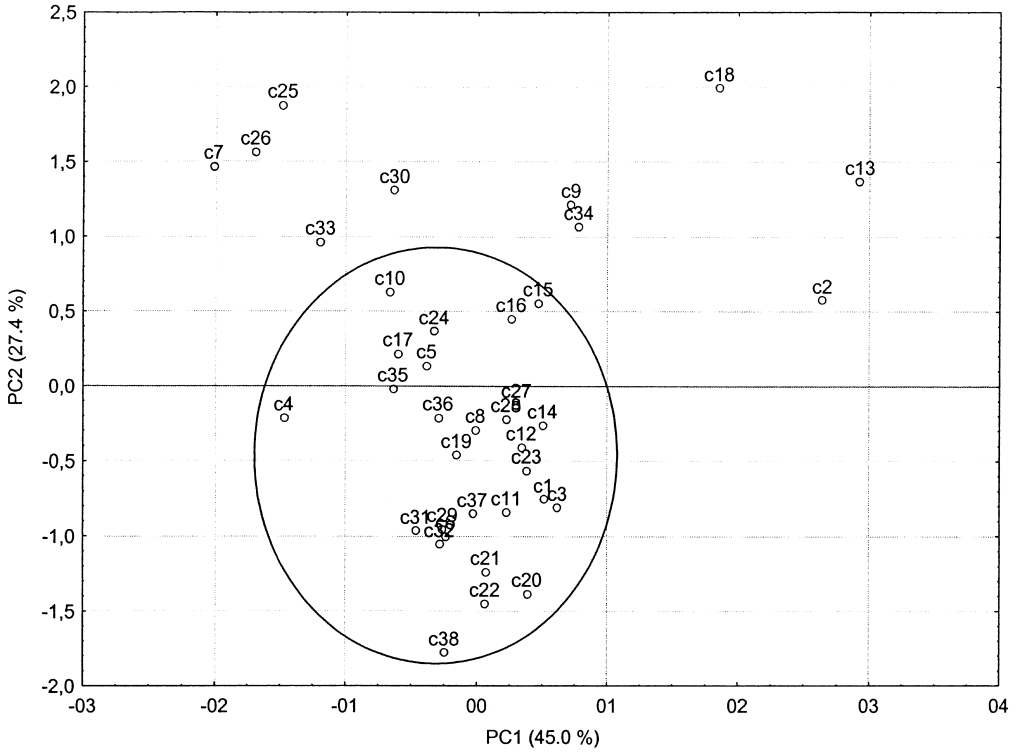


Fig. 4. Score plot of columns on the first and second principal components (PC1 and PC2).

MDCV 2 was totally separated from VM B and some unknown components that were coeluted with VM B, were also well separated. Moreover, DDCV and MDCV 1, which were coeluted in the previous systems, were also well separated.

High pH conditions lead to reduction of the column lifespan. Therefore, it was sought for a column, more compatible with high pH. Kromasil was chosen, as it is reported to remain stable up to pH 9.5. A less good separation was obtained when the mobile phase used for the Symmetry column was directly applied to the Kromasil column. However, the separation was improved when the amount of the buffer was increased up to 15%, but the analysis time was increased accordingly. By increasing the dioxane content up to 9%, a better separation was obtained in a shorter analysis time.

3.4. Optimization of the separation

The optimization of the separation at high pH was performed by experimental design and multivariate analysis using Modde 4.0 statistical graphic software. A central composite face centered (CCF) design was applied. In particular, the central composite design consisted of points of a two level full factorial design (2^k+n), which were augmented with $2k$ star points to enable this model to estimate the curvature response. $2^k+2k+n=27$ experiments were performed in duplicate, where $k=4$ is the number of parameters and $n=3$ is the number of central points. The central composite design permitted the response surface to be modeled by fitting a second-order polynomial model. The statistical relationship between a response Y and the experimental variables X_i, X_j,..., is of the following form:

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

where β is the regression coefficient and E, the overall experimental error. The linear coefficients for

the experimental variables, β_i and β_j describe their quantitative effects in the model. The cross product 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 [54,57].

The parameters examined were the amounts of dioxane and buffer in the mobile phase, the mobile phase pH and the temperature of the column. The chromatographic parameter settings in the experimental design are shown in Table 2. The individual and interaction parameter effects on the selectivity for the different pairs of compounds are summarized in Fig. 5. The plots consist of bars, which correspond to the regression coefficients. The magnitude of the variable effects is proportional to the regression coefficients. The 95% confidence limits are expressed by using error bars. A regression coefficient smaller than the error bar interval shows that the variation of the response caused by changing the variable is smaller than the experimental error. Therefore, the effect of variable change is considered insignificant when compared to the response. The coefficients of the terms in the model were estimated by partial least squares (PLS) method. The statistical analysis of the model gave R^2 values above 0.97 for all the selectivities. These R^2 values correspond to the fractions of the variation of the responses that can be explained by the model. The Q^2 values, which represent the fractions of the variation of the responses that can be predicted by the model, were all above 0.9. These values approach 1, which indicates the suitability of this model in predicting the optimum conditions.

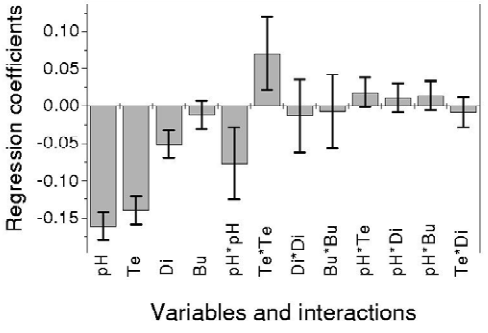
The results in Fig. 5 show that the separation of the pairs is principally influenced by the pH of the mobile phase and the column temperature. The effect of other parameters is less important. Fig. 6 shows the variation of selectivity as a function of pH and temperature. As can be seen, the selectivities obtained for pairs CDPm–CDPM, CDPM–DDCV and MDCV2–VM B are well above 1.3. The selection of the best conditions took into consideration the effect of different parameters on the separation of pairs DDCV–MDCV1, MDCV1–CDPinter and CDPinter–MDCV2. A pH of 7.7 and a temperature of 35 °C were found to give the best selectivity for all pairs. Moreover, in further experiments, an improvement of the separation was observed when the content of dioxane in the mobile phase was lowered down to 8%. As a gradient was needed to elute the more strongly retained AGLUV peak within acceptable time, the dioxane content was increased linearly to 30% after the elution of the MDCV 2 peak. Finally, the chromatographic conditions described in Fig. 7 were retained. As can be seen from this figure, the method allows a good separation of VM components. MDCV 2 and some unknown impurities that are not separated from the main component VM B with the official pharmacopoeia method [12,52,53] are now well separated. Some other known and unknown impurities are also well separated. This method should also allow a more correct quantitation of VM components, as they are now better separated. It is observed however that some impurity peaks are likely to still contain more than one compound. Separation of each single minor impurity peak was not achieved at this stage of study as VM proves to be a very complex substance. Compared to the separation obtained by CE [31], the LC method described here is more selective. However, the CE method offers the advantage of higher speed. In further investigations on the intermediate precision of the method, it was observed (see Fig. 8) that the use of a different batch of Kromasil column, combined with a different LC equipment, confirmed the good quality of separation previously achieved.

Table 2
Chromatographic parameter settings applied in the optimization investigation, corresponding to low (−), central (0) and high (+) levels

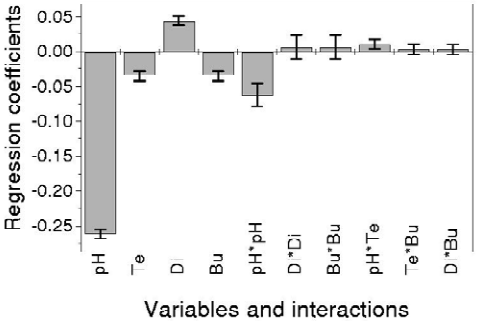
Parameter	Low value (−)	Central value (0)	High value (+)
pH	7.5	8.0	8.5
Temperature (°C)	25	30	35
Dioxane (%)	8.5	9	9.5
Buffer (%)	13	15	17

3.5. Robustness study

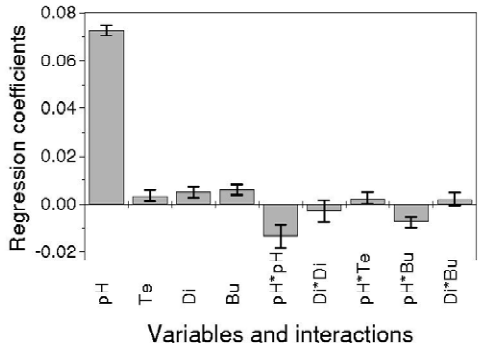
In this study, the effect of small changes of the most important chromatographic parameters on the gradient method was investigated. The parameters examined were the mobile phase pH, the column temperature and the content of dioxane in the mobile



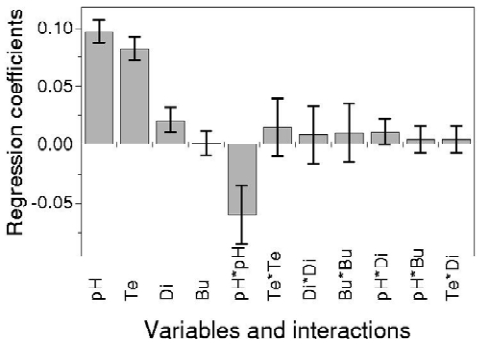
CDPm – CDPM



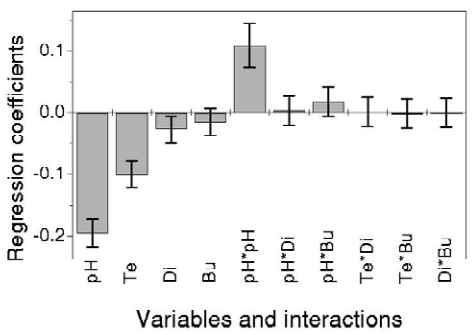
CDPM – DDCV



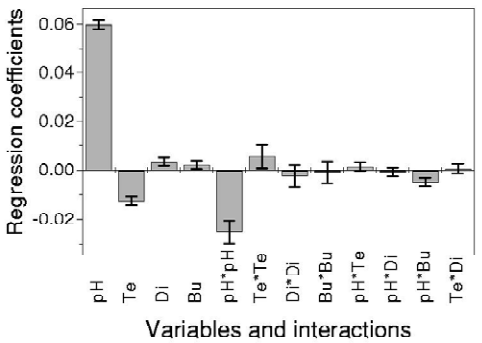
DDCV – MDCV1



MDCV1 – CDPinter



CDPinter – MDCV2



MDCV2 – VM B

Fig. 5. Regression coefficient plots of the selectivity for pairs CDPm–CDPM, CDP1M–DDCV, DDCV–MDCV1, MDCV1–CDPinter, CDPinter–MDCV2 and MDCV2–VM B. Te = Column temperature; pH = mobile phase pH; Di = dioxane content; Bu = buffer content.

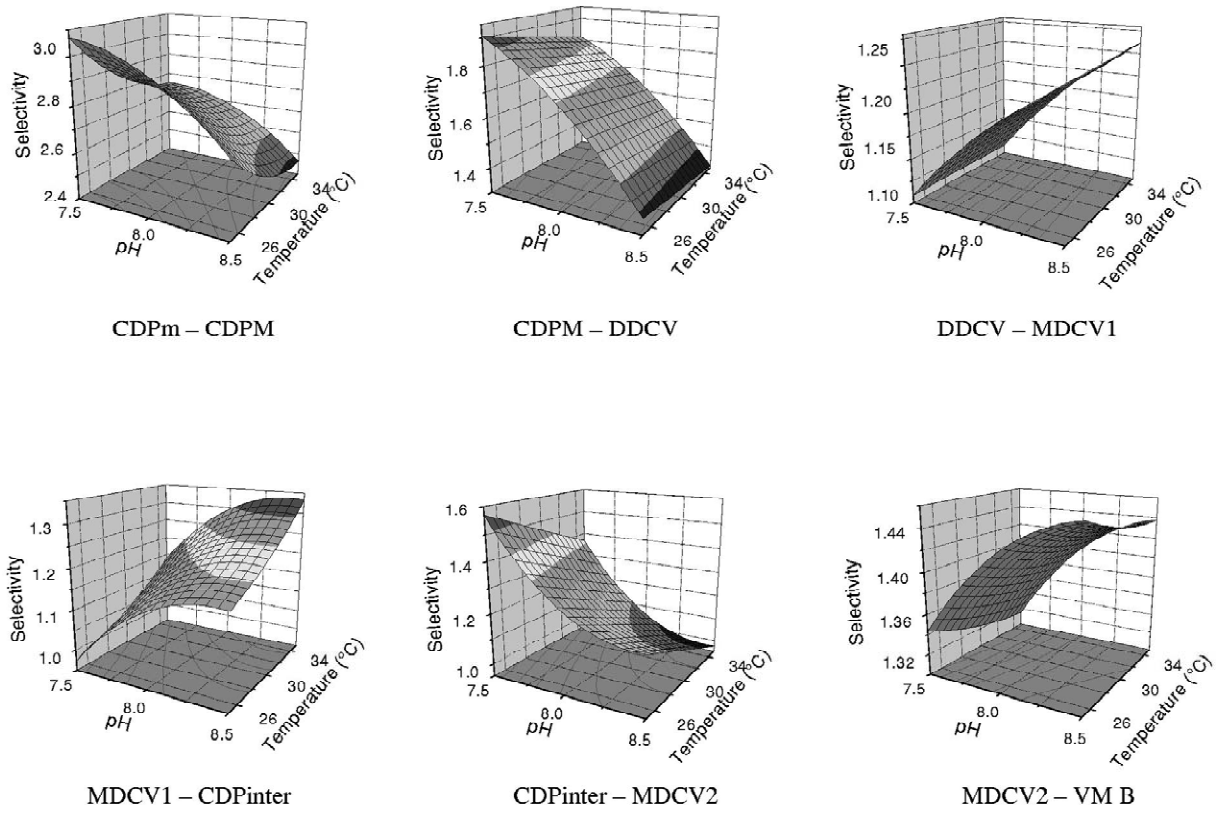


Fig. 6. Response surface plots of the selectivity for pairs CDPm–CDPM, CDPM–DDCV, DDCV–MDCV1, MDCV1–CDPinter, CDPinter–MDCV2 and MDCV2–VM as a function of the mobile phase pH and the column temperature.

phase during the isocratic part of the elution. Their effects on the selectivity for different pairs of compounds were evaluated by means of experimental design, using Modde 4.0 statistical graphic software. A central CCF design was applied. In particular, the central composite design consisted of points of a two-level full factorial design (2^k+n), which were augmented with $2k$ star points to enable this model to estimate the curvature response. $2^k+2k+n=17$ experiments were performed in duplicate, where $k=3$ is the number of parameters and $n=3$ is the number of central points. The settings applied in the experimental design for the pH, temperature and dioxane are shown in Table 3. The other chromatographic parameters were kept constant at their nominal values (see Section 2.3 and Fig. 7).

The individual and interaction parameter effects on the selectivity for pairs UNK5–CDPinter, CDPinter–UNK6, UNK6–MDCV2, MDCV2–UNK7, VM B–UNK9 and DESV–UNK12 are summarized in Fig. 9. The effects on the selectivity for other pairs are not discussed, as separation of those pairs was only very slightly affected by these parameter changes. It is observed that the separation under the examined conditions is principally influenced by the pH of the mobile phase. The effects of dioxane and temperature are insignificant, except for pairs VM B–UNK9 and DESV–UNK12. For the latter, the effect of temperature is the most important of all. Fig. 10 shows the variation of the selectivity as a function of pH and dioxane content for pairs UNK5–CDPinter, CDPinter–UNK6, UNK6–MDCV2, MDCV2–UNK7, VM B–UNK9 and as a function of temperature and dioxane content for pair DESV–UNK12. It is observed that a decrease of pH down to 7.5 may decrease the selectivity for pairs UNK5–CDPinter and UNK6–MDCV2. On the other hand, an increase of pH up to 7.9 may decrease the

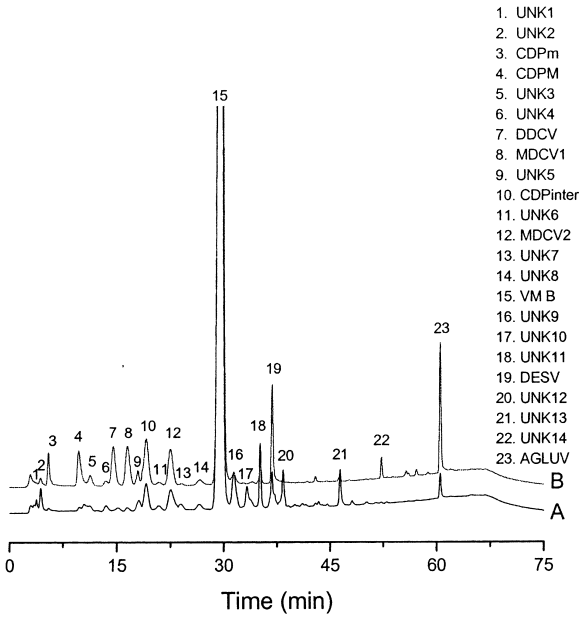


Fig. 7. Typical chromatograms of 2 mg/ml solution of (A) VM·HCl commercial sample VM·HCl) and a purified VM·HCl sample spiked with its known impurities. Column: Kromasil (250×4.6 mm I.D.), 5 μm, maintained at 35 °C. Mobile phases: dioxane–ammonium formate buffer, pH 7.7–water (A) (7:15:78, v/v) and (B) (30:15:55, v/v/v). Gradient program: 0–20 min, 5% of B (isocratic); 20–55 min, 5 to 100% of B (linear gradient); 55–60 min, 100% of B (isocratic); 60–70 min, 100 to 5% of B (linear gradient); 70–85 min, 5% of B (isocratic). Detection: UV at 280 nm; flow-rate: 1.0 ml/min; injection volume: 20 μl.

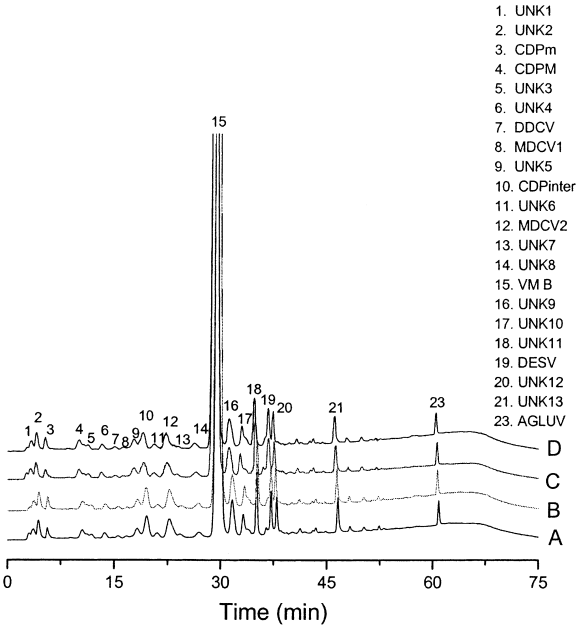


Fig. 8. Effect of different batches of Kromasil column and different LC equipment on the separation of VM components: (A) old Kromasil column, equipment I; (B) brand-new Kromasil column, equipment I, (C) old Kromasil column, equipment II and (D) brand-new Kromasil column, equipment II. A 2 mg/ml solution of VM·HCl commercial sample was analyzed. All the chromatographic conditions are as described in Fig. 7.

selectivity for pairs MDCV2–UNK7 and VM B–UNK9. Furthermore, a decrease of temperature down to 33 °C may decrease the selectivity for pair DESV–UNK12. It can be concluded that the pH should be monitored carefully to ensure the best separation for the known as well as for the unknown components of the VM complex. Small changes of temperature and dioxane content do not have a harmful effect on the separation except for pair DESV–UNK12, which is not well separated at lower temperature.

3.6. Quantitative aspects

3.6.1. Calculations

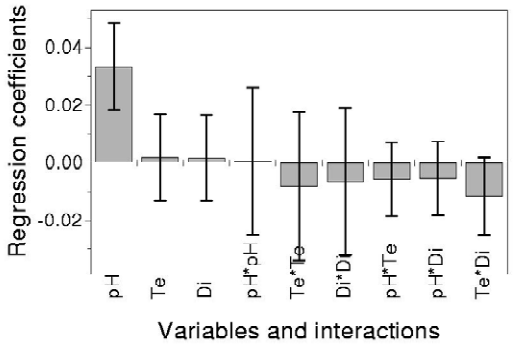
For quantitation of VM B and its related substances an external standard approach is preferred. This needs the elaboration of a standard, which was not available. Results can be generated by comparison between peak areas in chromatograms obtained with the sample solutions and the main peak area in chromatograms obtained with standard solutions.

The content of related substances is calculated using the following equation:

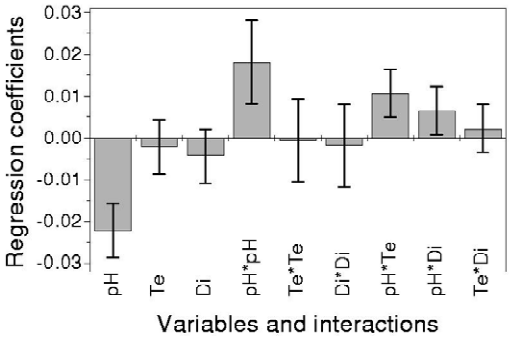
$$\text{Content of individual related substance (\%)} = (\text{area}_i)/(\text{area VM B std 1\%})$$

Table 3
Chromatographic parameter settings applied in the robustness investigation, corresponding to low (−), central (0) and high (+) levels

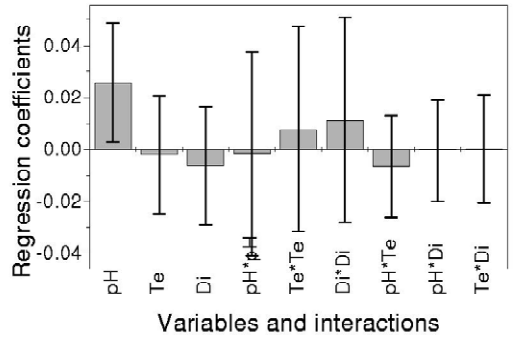
Parameter	Low value (−)	Central value (0)	High value (+)
pH	7.5	7.7	7.9
Temperature (°C)	33	35	37
Dioxane (%)	7.80	8.15	8.50



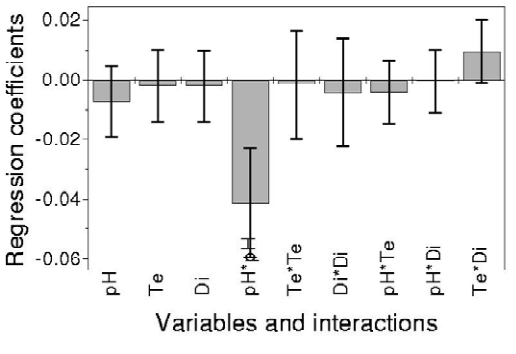
UNK5 – CDPinter



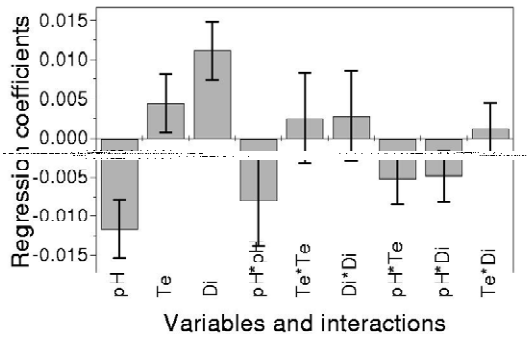
CDPinter – UNK 6



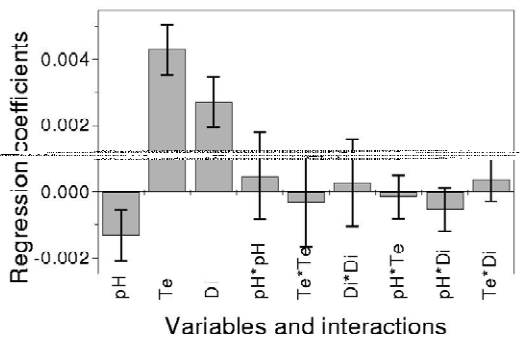
UNK6 – MDCV2



MDCV2 – UNK7



VM B – UNK9



DESV – UNK12

Fig. 9. Regression coefficient plots of the selectivity for pairs UNK5–CDPinter, CDPinter–UNK6, UNK6–MDCV2, MDCV2–UNK7, VM B–UNK9 and DESV–UNK12. Te = Column temperature; pH = mobile phase pH; Di = dioxane content.

Table 4
Repeatability data for VM and its impurities

	VM B	CDPm	CDPM	DDCV	MDCV1	CDPinter	MDCV2	DESV	AGLUV	No. experiments (*n*)
Level (%)	80	0.8	1.5	2	2	2.5	2	2	2	
Intra-day RSD (%)	0.16	0.67	0.59	0.35	0.21	0.28	0.31	0.37	0.45	6
Inter-day RSD (%)	0.25	0.88	0.74	0.47	0.38	0.34	0.46	0.44	0.50	18 (3 days)

weighings of the sample injected in triplicate in a single day gave an RSD ($n=9$) value of 0.31 for the content of VM B. An RSD ($n=27$) value of 0.43 for the content of VM B was obtained assaying the sample on three consecutive days. An intermediate precision study was performed using a new column and a different LC equipment. Three weighings of the same commercial sample injected in triplicate in a single day gave an RSD ($n=9$) value of 0.54 for the content of VM B. Combining the results for content of VM B obtained with equipment I and II, an RSD ($n=18$) value of 0.9 was obtained. These results demonstrate the good precision of the method. The linearity was checked by separate analysis of VM components. Each compound was assayed in triplicate over the concentration ranges 25–125% and 0.1–2.5%, respectively, for the main component and for the impurities. All the percentages were calculated with respect to the amount of VM injected (40 μg = 100%) in an analysis as described in the Experimental section. Good linearity was observed for the different compounds. The correlation coefficients (R^2) of the calibration curves were all above 0.998. The limits of quantitation (LOQs) (corresponding to a signal-to-noise ratio of 10) and the corresponding RSD values are presented in Table 5. Note that the LOQ values observed for DESV and AGLUV are low compared to that of other components. This is because peaks were sharper as the amount of dioxane in the mobile phase increased with gradient elution.

An LOQ of 0.1% (RSD = 7.9%, $n=6$), calculated versus the amount of substance brought in the capillary, was reported for the main component VM B in the CE method described by Kang et al. [31]. Comparing these data to those described in this study, the LC method developed here shows a better sensitivity.

3.7. System suitability

A system suitability test is proposed. It is developed so that no sample of related substances is needed. The system suitability solution is prepared by dissolving 10 mg of VM·HCl sample in 20 ml of mobile phase and heating this solution at 65 °C for 24 h. Under these conditions, VM B is partially converted into the two isomers of CDP (CDPm and CDPM). Peak-to-valley ratio between the main peak and the impurity on its tail and resolution between CDPm and CDPM were established as system suitability parameters. The limit for the peak-to-valley ratio is 6. It is defined as H_p/H_v with H_p = height above the extrapolated baseline of peak due to

Table 5
Limits of quantitation (LOQs) and corresponding RSD values for VM and its impurities

	VM B	CDPm	CDPM	DDCV	MDCV1	CDPinter	MDCV2	DESV	AGLUV
LOQ									
Percent (%)	0.02	0.02	0.03	0.03	0.03	0.04	0.03	0.01	0.01
Mass on column (ng)	8	8	12	12	12	16	12	4	4
RSD (%, $n=6$)	8	11	11	8	8	10	9	8	8

The percentages are calculated versus the amount of VM injected on the column in an analysis as described in the Experimental section (40 μg = 100%).

the impurity and H_v = height above the extrapolated baseline at the lowest point of the curve separating the peaks due to the impurity and VM B. The limit for the resolution between CDPm and CDPM is 6. When these criteria are not met, the dioxane content in the mobile phase should be adjusted slightly.

4. Conclusion

The proposed LC method allows good separation of VM components, which is much better than that obtained with existing methods. This method shows better selectivity and higher efficiency than the Ph. Eur and USP method, especially towards MDCV 2 and some unknown impurities that are now separated from the main component VM B. The method is also linear, repeatable and sensitive and it is a suitable alternative to official methods for the analysis of VM. Investigation of the method robustness showed that the pH has the most important effect and should be monitored carefully, especially as the ammonium formate solution has very low buffer capacity at the pH used.

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