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# Mobile Phase Optimization Method for Steroids Separation

**Carmen Elena Stoenoiu[1], Sorana Daniela Bolboacă[2], Lorentz Jäntschi[1]**

[1] Technical University of Cluj-Napoca, Cluj-Napoca, Romania
[2] „Iuliu Haţieganu“University of Medicine and Pharmacy, Cluj-Napoca, Romania

***Abstract*** *- The paper presented a mathematical model developed for optimization of mobile phase composition in thin layer chromatography applied on steroids separation. The proper solvents system was experimental identify as being a mixture of chloroform, cyclohexan, and methyl-ethyl-cetone. An original mathematical model was developed and used in order to identify the composition of mobile phase. Starting with the mathematical model and with the optimization procedure a computer program has been developed. The proposed model is able to optimize simultaneously many mobile phases with respect of the shortest analysis time and of the selectivity of compounds. The efficiency of the mathematical and optimization models is demonstrated on a sample of five androstane isomers.*
***Key words:*** *Androstane Isomers, Thin-Layer Chromatography (TLC), Mobile Phase Optimization*

## Introduction

The steroids hormones are natural or synthetic compounds with physiological activities derived from cholesterol. With a single exception, represented by vitamin D, they have a cyclopentanophenanthrene skeleton and atomic numbering system as cholesterol (see Figure 1 [1], note that the carbon 18 and above can be absent).

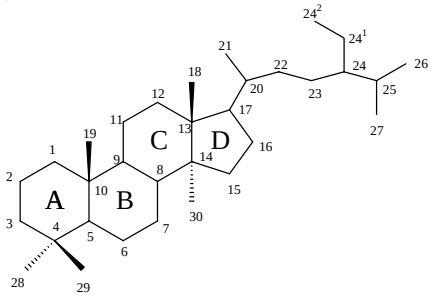


**Figure 1. Steroid nucleus**

The term “steroids” has been introduced by Callow R.K. & Young F. G. in 1936 [2] for the group of compounds comprising the sterols, bile acids, heart poisons, saponins, and sex hormones. The most common categories of steroids include: anabolic steroids (class of steroids that increase muscle and bone synthesis [3]), corticosteroids (glucocorticosteroids – regulate aspects of metabolism and immune functions [4,5]; mineralocorticoids – maintain blood volume and control renal excretion of electrolytes [6]); sex steroids (androgens, estrogens, and progestagens – produce sex differences and support reproduction [7]); phytosterols (steroids that naturally occur in plants [8]); and ergosterols (steroids that occur in fungi – includes some Vitamin D supplements [9]). Androstane is a steroid hydrocarbon from which androgens are derived and has the generic structure as is shown in Figure 2.

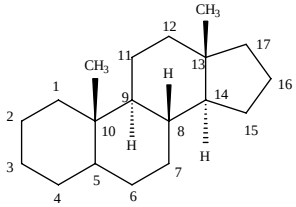


**Figure 2. Androstane generic structure**

Chromatographic analysis, defined as techniques used for the separation of a mixture of compounds by their distribution between two phases, was introduced in 1901 by Mikhail Semyonovich Tsvet [10]. Thin layer chromatography is the chromatographic technique used since 1963 [11] for separation chemical compounds including of steroids. The method is still used in separation of compound

because of its advantages comparing with column chromatography [12]: (1) single use of the layer simplifies sample preparation procedures; (2) simplicity of development by dipping the plate into a mobile phase in a chamber; (3) high sample through-put with low operating cost because multiple samples can be run simultaneously with standards on a single plate using a very low volume of solvent; (4) selective and sensitive post-chromatographic detection and identification; (5) visual observation and direct recording of the entire chromatogram including all components, the origin, and the mobile phase front; and (6) the ability to repeat detection and quantification steps under different conditions. The most delicate problem in steroids separation is to chouse the optimum mixture of solvents in order to obtain the maximum separation.

A sample of six androstane isomers was previous investigated for identification of optimum mobile phase [13]. The best experimental results were obtained by the solvent system with the following compounds: Chloroform – Cyclohexan - Methyl-Ethyl-Cetone. The obtained experimental data are presented in Table 1. The best results expressed as mobile phase composition and objective function obtained by using Simplex, and Prisma methods are presented in Table 2.

**Table 1. Experimental data obtained for separation of androstane isomers**

| Exp. No. | Composition of mobile phase Chloroform : Cyclohexan : Methyl-Ethyl-Cetone | Amount of information | | | | | Width of the compound spot | | | | | Elu-ent |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $I_1$ | $I_2$ | $I_3$ | $I_4$ | $I_5$ | $w_1$ | $w_2$ | $w_3$ | $w_4$ | $w_5$ | |
| 1 | 33 : 33 : 33 | 5.51 | 6.10 | 6.09 | 2.45 | 3.32 | 0.50 | 0.31 | 0.19 | 0.26 | 0.64 | 7.76 |
| 2 | 10 : 10 : 80 | 7.43 | 7.92 | 7.91 | 6.14 | 6.48 | 0.51 | 0.21 | 0.19 | 0.31 | 0.32 | 8.84 |
| 3 | 10 : 80 : 10 | 2.05 | 2.97 | 2.76 | 0.15 | 0.28 | 0.27 | 0.21 | 0.22 | 0.23 | 0.15 | 9.64 |
| 4 | 80 : 10 : 10 | 3.45 | 5.03 | 4.59 | 0.58 | 1.33 | 0.54 | 0.31 | 0.29 | 0.21 | 0.35 | 8.91 |
| 5 | 50 : 50 : 0 | 0.53 | 0.96 | 0.75 | 0.15 | 0.20 | 0.41 | 0.31 | 0.22 | 0.25 | 0.25 | 9.04 |
| 6 | 50 : 0 : 50 | 6.23 | 0.85 | 6.89 | 4.00 | 4.82 | 0.56 | 0.31 | 0.24 | 0.29 | 0.51 | 8.85 |
| 7 | 0 : 50 : 50 | 6.44 | 6.83 | 6.80 | 5.64 | 4.10 | 0.52 | 0.21 | 0.11 | 0.21 | 0.82 | 8.55 |
| 8 | 100: 0 : 0 | 0.92 | 1.89 | 2.47 | 0.24 | 0.26 | 0.43 | 0.32 | 0.21 | 0.22 | 0.22 | 8.77 |
| 9 | 0 : 100 : 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.46 | 0.32 | 0.15 | 0.24 | 0.21 | 8.41 |
| 10 | 0 : 0 :100 | 8.26 | 8.42 | 8.34 | 7.29 | 7.00 | 0.31 | 0.16 | 0.15 | 0.21 | 0.47 | 8.93 |

I = amount of information; w = width of the compound spot; i = 1…4 (1 = 5α-androstane-3β-ol, 2 = 5α-androstane-3α-ol, 3 = 5α-androstane-17β-ol, 4 = 5β-androstane-3α,17β-diol, 5 = 5β-androstane-3β,17β-diol)

**Table 2. Mobile phase composition and corresponding values for objective function according with applied method**

| No. | Method | Chloroform : Cyclohexan : Methyl-Ethyl-Cetone | $F_{ob}$ |
|---|---|---|---|
| 1. | Simplex | 10.5 : 5.1 : 4.4 | 13.20 |
| 2. | Prisma | 50 : 30 : 20 | 11.34 |

Starting with the experience obtained in optimization of the mobile phase in High-Performance Thin-Layer Chromatography of a sample of benzodiazepines [14,15], the aim of the present research was to develop and to assess a mathematical model useful in separation of androstane isomers.

## Material and Method

*Androstane Isomers*

A sample of five androstane was the material of the present study. The following isomers were included into analysis: 5α-androstane-3β-ol, 5α-androstane-3α-ol, 5α-androstane-17β-ol, 5β-androstane-3α,17β-diol, 5β-androstane-3β,17β-diol (Figure 3).

*Mathematical Model and Optimization Procedure*

The quantitative measure of a chromatographic parameter put in a mixture of three solvents depends on the composition of mobile phase. The dependence equation could be one with six or with seven parameters (Eq.(1) and Eq.(2)):

$$M6(x_1,x_2,x_3) = a_1x_1 + a_2x_2 + a_3x_3 + a_4x_1x_2 + a_5x_1x_3 + a_6x_2x_3 \quad (1)$$

$$M7(x_1,x_2,x_3) = a_1x_1 + a_2x_2 + a_3x_3 + a_4x_1x_2 + a_5x_1x_3 + a_6x_2x_3 + a_7x_1x_2x_3 \quad (2)$$

where $x_1$, $x_2$, $x_3$ are molar fraction of the three solvents ($x_1 + x_2 + x_3 = 1$), *M6* and *M7* are

estimators and then predictors of choused chromatographic parameter, and $a_1$, $a_2$, $a_3$, $a_4$, $a_5$, $a_6$, $a_7$ are coefficients first determined based on the best estimation of choused chromatographic parameter and then used in prediction.

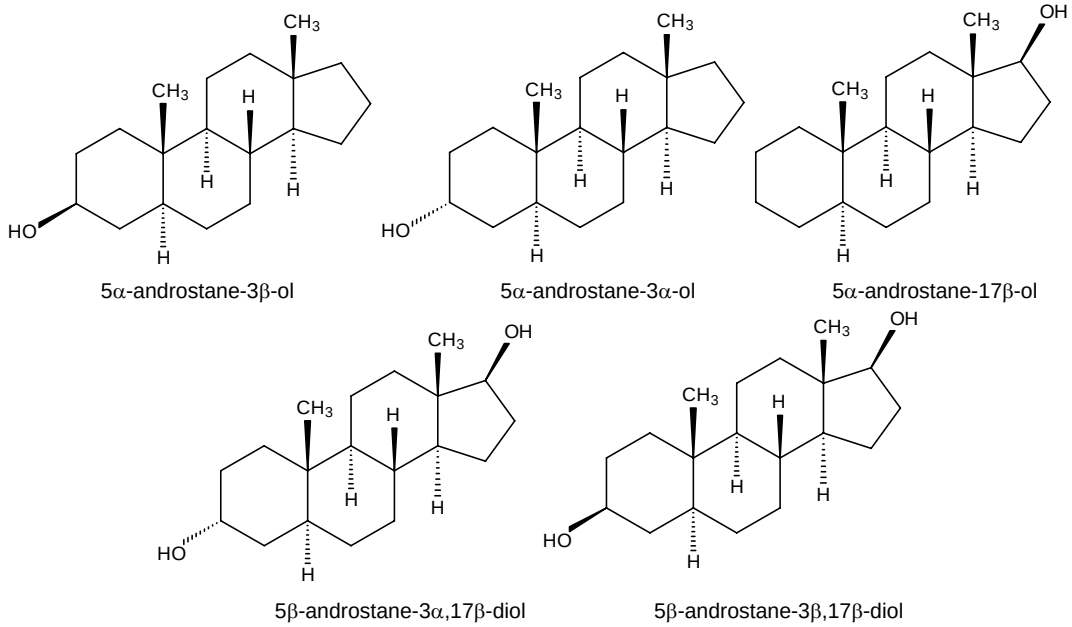


**Figure 3. Androstane isomers include into analysis**

The following chromatographic parameters were modeled starting from Eq.(1) and Eq.(2):

$$F_{ob}(e,m) = \Sigma j a_j F_j(Sm(e),Inf(e,m),RSA(e),RRP(e)) \quad (3)$$

where $F_{ob}$ is an objective function which characterized the separation with the eluent $e$ in report with selection of coefficients $a_j$, $1 \leq j \leq 4$, $F_j$ are functions that contain an expression of four parameters, $a_j$ are coefficients choused arbitrary or through of a defined ponderate mathematical relation of the $F_j$ functions and respectively of the number of equidistant intervals $m$, *RSA* is the mean of separation resolution by using the eluent $e$, and *RRP* is the ponderate product with the mean of the resolutions used in separation with the eluent $e$.

$$RS(i,j,e) = 2\cdot(l(i)-l(j))/(w(i)+w(j)) \quad (4)$$

where $i$, $j$ are two separation compounds, $w(i)$ and $w(j)$ are the width of the compound's spots, and *RS* is the matrix of calculated resolution for separation of the compound $i$ by the compound $j$.

$$RF(i,e) = l(i)/l(e) \quad (5)$$

where $i$ is one of the separation compounds, $e$ is the eluent used as mobile phase, $l(i)$ is the coordinate at which the $e$ eluent had migrated, $l(e)$ is the coordinate at which the eluent had migrated, and *RF* is the series of retention factor of the separation compounds for the eluent $e$.

By application of one of the above describe equations (Eq.(3)-Eq.(5)) on a series of $p$ experiments, there result a $M_{ob}$ matrix with one ore more than one rows (one row for each experiment). The elements of $M_{ob}$ matrix represent the values of chromatographic parameter that is modeled by using Eq.(1) or Eq.(2). The optimization model has a unique solution for $p \geq 6$ (Eq.(1)), and for $p \geq 7$ (Eq.(2)), respectively.

A system can be build for each row from $M_{ob}$ matrix with $p$ linear equations (where p = 6 (Eq.(1), and 7 (Eq.(2)), respectively):

$$M_{ob}(j) = a_1x_1 + a_2x_2 + a_3x_3 + a_4x_1x_2 + ... \quad (6)$$

where $x_i$ are the molar fractions of each solvent ($i = 1, 2, 3$) that enter into the composition of the $e_j$ eluent ($j = 1, 2, \ldots, p$).

To the above describe system (Eq.(6)) the least squared method was applied for construction of the system with unique solution (*MMCP*); the solution is obtained by applying the following formula:

$$MMCP(k,0) = M2(MOB,A(k)),$$
$$MMCP(k,l) = M2(A(k),A(l)) \quad (7)$$

where $(k,0) = 1, 2, \ldots, 6$ for Eq.(1) and $(k,0) = 1, 2, \ldots, 7$ for Eq.(2), $A(k)$ are the series of terms known from Eq.(6), $M2$ is the mean calculated for the product of $M_{ob}$ series and $A(k)$, and

*MMCP* is the extended matrix of system of the linear equations which is used in determination of the coefficients $a_k$.
The Gaussian method was applied to found the solution for Eq.(7). The found solutions for the systems from Eq.(1) and Eq.(2) are:

$$A0 = (a_{01}, a_{02}, \ldots, a_{06}) \quad \text{- for Eq.(1)}$$
$$A0 = (a_{01}, a_{02}, \ldots, a_{07}) \quad \text{- for Eq.(2)} \qquad (8)$$

The *A0* coefficients are used in prediction of the chromatographic parameter of interest by using one of the equations Eq.(1) or Eq.(2). For example if *Y* is the choused chromatographic parameter, the $M_{ob}$ matrix (the predictor of *Y*), as well as the estimator of *Y*, had more than one row. If *z* is the number of $M_{ob}$ matrix rows (and implicit the number of predictors) then the estimator of choused chromatographic parameter $\hat{Y}$ is:

$$\hat{Y} = (\hat{y}_1, \ldots, \hat{y}_z) \qquad (9)$$

The optimum can be obtained by application of a maximization or minimization function (as is for example the characterization of a separation of many compounds through the worst separation of two compounds):

$$\hat{y}_o = opt(\hat{Y}), \text{ where } opt = \text{"max" or } opt = \text{"min"} \qquad (10)$$

Moving through all domains of possible values for the composition of the mobile phase, the optimum point was identified, this being the optimum composition of mobile phase ($x_1$, $x_2$, $x_3$):

$$(\cdot,\cdot,\cdot) \mid \hat{Y}(\cdot,\cdot,\cdot) = opt\{ \hat{Y}(i/100,j/100,k/100) \mid i=0..100, \; j=0..100-i, \; k=100-i-j\} \qquad (11)$$

**Results**

The mathematical model has been integrated into a program dedicated to the optimization of the mobile phase composition. The program is freely available from the following URL:
http://vl.academicdirect.org/molecular_dynamics/mobile_phase_opt/
The generic equations that proved to obtained performances in optimization of the mobile phase, according with the chromatographic parameter of interest and composition of optimum mobile phase (Chloroform – Cyclohexan – Methyl-Ethyl-Cetone) are presented in Table 3.
The values of the retention factor obtained experimentally and by mathematical model, respectively are presented in Table 4.
The correlation coefficient between experimental and optimized retention factor, associated 95% confidence interval, the squared correlation coefficient are presented in Table 5.

**Table 3. Results of optimization procedure**

| No. | Parameter | Equation | Optimum mobile phase |
|---|---|---|---|
| 1. | Retention factor (Δrf) | $\Delta rf = a_1x_1+a_2x_2+a_3x_3+a_4x_1x_2+a_5x_1x_3+a_6x_2x_3+a_7x_1x_2x_3$ | 60 : 13 : 27 |
| 2. | Separation Resolution (All_RS) | $All\_RS = a_1x_1+a_2x_2+a_3x_3+a_4x_1x_2+a_5x_1x_3+a_6x_2x_3+a_7x_1x_2x_3$ | 64 : 0 : 36 |
| 3. | Objective function ($F_{ob}$) | $F_{ob} = a_1x_1+a_2x_2+a_3x_3+a_4x_1x_2+a_5x_1x_3+a_6x_2x_3+a_7x_1x_2x_3$ | 64 : 0 : 36 |

**Table 4. Experimental and optimized retention factor for androstane isomers**

| Exp.No. | 5α-androstane 3β-ol | | 5α-androstane 3α-ol | | 5α-androstane 17β-ol | | 5β-androstane 3α,17β-diol | | 5β-androstane 3β,17β-diol | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Exp. | MathM. | Exp. | MathM. | Exp. | MathM. | Exp. | MathM. | Exp. | MathM. |
| 1 | 0.7101 | 0.7073 | 0.7861 | 0.8477 | 0.7848 | 0.7939 | 0.3157 | 0.2854 | 0.4278 | 0.4136 |
| 2 | 0.8405 | 0.9097 | 0.8959 | 0.8508 | 0.8948 | 0.9513 | 0.6946 | 0.7154 | 0.7330 | 0.7186 |
| 3 | 0.2127 | 0.2310 | 0.3081 | 0.3080 | 0.2863 | 0.2643 | 0.0156 | 0.1082 | 0.0290 | 0.0999 |
| 4 | 0.3872 | 0.3124 | 0.5645 | 0.3255 | 0.5152 | 0.4387 | 0.0651 | 0.0914 | 0.1493 | 0.1588 |
| 5 | 0.0586 | 0.0752 | 0.1062 | 0.1572 | 0.0830 | 0.1082 | 0.0166 | 0.0000 | 0.0221 | 0.0033 |
| 6 | 0.7040 | 0.7062 | 0.0960 | 0.1597 | 0.7785 | 0.7818 | 0.4520 | 0.4466 | 0.5446 | 0.5497 |
| 7 | 0.7532 | 0.7294 | 0.7988 | 0.7956 | 0.7953 | 0.7833 | 0.6596 | 0.6357 | 0.4795 | 0.4674 |
| 8 | 0.1049 | 0.1475 | 0.2155 | 0.3334 | 0.2816 | 0.3221 | 0.0274 | 0.0205 | 0.0296 | 0.0280 |
| 9 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0100 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| 10 | 0.9250 | 0.8869 | 0.9429 | 0.9522 | 0.9339 | 0.8999 | 0.8163 | 0.8125 | 0.7839 | 0.7957 |

Exp. = retention factor obtained from experimental data;
MathM. = retention factor obtained by mathematical model

**Table 5. Results of correlation between experimental and optimized values for retention factor**

| Androstane isomers | r | 95%$CI_r$ | $r^2$ |
|---|---|---|---|
| 5α-androstane 3β-ol | 0.9934 | [0.9714-0.9985] | 0.9869 |
| 5α-androstane 3α-ol | 0.9648 | [0.8536-0.9918] | 0.9307 |
| 5α-androstane 17β-ol | 0.9939 | [0.9734-0.9986] | 0.9879 |
| 5β-androstane 3α,17β-diol | 0.9943 | [0.9750-0.9986] | 0.9886 |
| 5β-androstane 3β,17β-diol | 0.9966 | [0.9851-0.9992] | 0.9932 |

r = correlation coefficient;
95% $CI_r$ = 95% confidence intervals for correlation coefficient;
$r^2$ = squared correlation coefficient

The graphical representation of the retention factor estimated through optimization versus retention factor experimentally obtained for 5β-androstane-3β,17β-diol compound is presented in Figure 4.

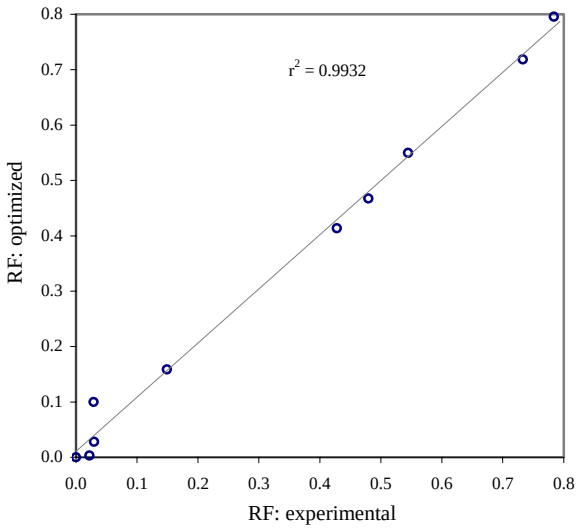


**Figure 4. Optimized versus experimental retention factor for 5β-androstane-3β,17β-diol**

Applying the mathematical model to the experimental data the graphical representation of the retention factor at optimum mobile phase (Chloroform – Cyclohexan – Methyl-Ethyl-Cetone = 60 : 13 : 27) the graphical representation presented in Figure 5 was obtained. The spots of dark color indicate the optimum mobile phase obtained by the mathematical model (Chloroform – Cyclohexan = 60 : 13).

The values of the resolution for separation obtained from the experimental data and respectively by the mathematical model for the optimum mobile phase (Chloroform – Cyclohexan – Methyl-Ethyl-Cetone = 60 : 0 : 36) are presented in Table 6. The correlation coefficient obtained between experimental and optimized values, associated 95% confidence intervals and squared correlation coefficients are presented in Table 7.

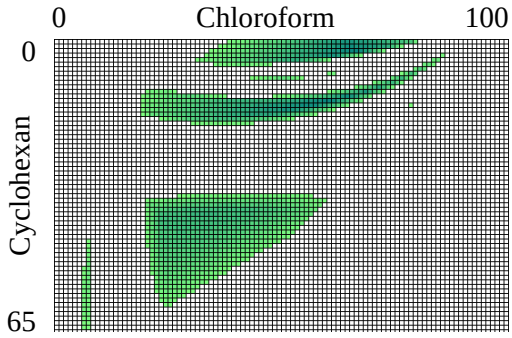


**Figure 5. The plot of the retention factor obtained through optimization procedure**

The squared correlation coefficient between objective function calculated based the experimental data and objective function calculated by using the mathematical model for the optimum mobile phase (Chloroform – Cyclohexan – Methyl-Ethyl-Cetone = 60 : 0 : 36) was equal with 0.8921 (95%$CI_r$ = [0.7769 – 0.9871]). Applying the mathematical model on experimental data presented in Table 1, the diagrams of the objective function obtained for presented in Figure 6 was obtained (the composition of the used optimum mobile phase was Chloroform – Cyclohexan – Methyl-Ethyl-Cetone = 60 : 0 : 36).

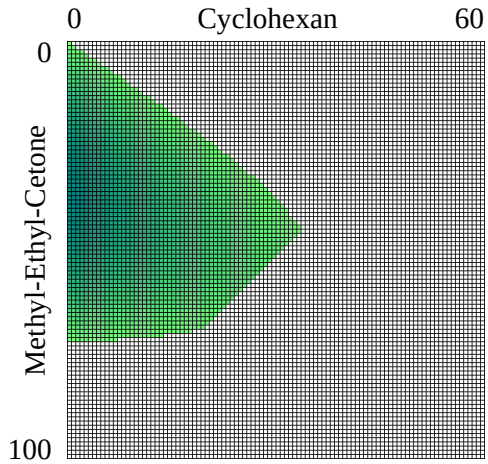


**Figure 6. The diagram of the objective function (Cyclohexan & Methyl-Ethyl-Cetone)**

The optimum mobile phases appear on the diagram as darkest spots (Cyclohexan – Methyl-Ethyl-Cetone = 0 : 36), the color becoming lighter as the distance from the optimum mobile phase is increased. In the region where the spots are darkest, the composition of the optimum mobile phase can be established according with the value of the objective function ($F_{ob}$).

**Table 6. Experimental and optimized separation resolution for androstane isomers**

| Separation Resolution | | Exp.No. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 5α-androstane-3β-ol & 5α-androstane-3α-ol | Exp. | 1.4568 | 1.3611 | 3.8333 | 3.7176 | 1.1944 | 12.3678 | 1.0685 | 2.5867 | 0.0000 | 0.6809 |
| | MathM. | 1.5995 | 2.8743 | 1.0491 | 4.3299 | 1.7656 | 11.7357 | 1.3874 | 2.2069 | 1.5223 | 0.0000 |
| | E-M | -0.1427 | -1.5132 | 2.7842 | -0.6123 | -0.5712 | 0.6321 | -0.3189 | 0.3798 | -1.5223 | 0.6809 |
| 5α-androstane-3β-ol & 5α-androstane-17β-ol | Exp. | 1.6812 | 1.3714 | 2.8980 | 2.7470 | 0.6984 | 1.6500 | 1.1429 | 4.8438 | 0.0000 | 0.3478 |
| | MathM. | 2.0585 | 1.0231 | 1.1217 | 3.1298 | 0.9909 | 1.5426 | 1.6400 | 4.5318 | 0.8971 | 0.4453 |
| | E-M | -0.3773 | 0.3483 | 1.7763 | -0.3828 | -0.2925 | 0.1074 | -0.4971 | 0.312 | -0.8971 | -0.0975 |
| 5α-androstane-3β-ol & 5β-androstane -3α,17β-diol | Exp. | 8.0526 | 3.1463 | 7.6000 | 7.6533 | 1.1515 | 5.2471 | 2.1918 | 2.0923 | 0.0000 | 3.7308 |
| | MathM. | 9.1778 | 4.6253 | 3.5548 | 5.0261 | 2.7285 | 5.2772 | 2.6190 | 3.2725 | 1.9742 | 2.6115 |
| | E-M | -1.1252 | -1.479 | 4.0452 | 2.6272 | -1.577 | -0.0301 | -0.4272 | -1.1802 | -1.9742 | 1.1193 |
| 5α-androstane-3β-ol & 5β-androstane -3β,17β-diol | Exp. | 3.8421 | 2.2892 | 8.4286 | 4.7640 | 1.0000 | 2.6355 | 3.4925 | 2.0308 | 0.0000 | 3.2308 |
| | MathM. | 5.0588 | 3.3510 | 3.4511 | 3.0638 | 2.5547 | 2.4993 | 4.2739 | 2.6682 | 2.4726 | 2.3214 |
| | E-M | -1.2167 | -1.0618 | 4.9775 | 1.7002 | -1.5547 | 0.1362 | -0.7814 | -0.6374 | -2.4726 | 0.9094 |
| 5α-androstane-3α-ol & 5α-androstane-17β-ol | Exp. | 0.0400 | 0.0500 | 0.9767 | 1.4667 | 0.7925 | 21.9636 | 0.1875 | 2.1887 | 0.0000 | 0.5161 |
| | MathM. | 0.0000 | 3.5802 | 0.0000 | 4.7737 | 0.6087 | 20.3429 | 0.0000 | 0.6302 | 1.1904 | 0.0000 |
| | E-M | 0.04 | -3.5302 | 0.9767 | -3.307 | 0.1838 | 1.6207 | 0.1875 | 1.5585 | -1.1904 | 0.5161 |
| 5α-androstane-3α-ol & 5β-androstane -3α,17β-diol | Exp. | 12.8070 | 6.8462 | 12.8182 | 17.1154 | 2.8929 | 10.5000 | 5.6667 | 6.1111 | 0.0000 | 6.1081 |
| | MathM. | 15.2315 | 8.5199 | 6.2142 | 10.8550 | 5.8671 | 11.1565 | 6.4194 | 8.9897 | 3.0710 | 4.5436 |
| | E-M | -2.4245 | -1.6737 | 6.604 | 6.2604 | -2.9742 | -0.6565 | -0.7527 | -2.8786 | -3.071 | 1.5645 |
| 5α-androstane-3α-ol & 5β-androstane -3β,17β-diol | Exp. | 5.8526 | 5.4340 | 14.9444 | 11.2121 | 2.7143 | 9.6829 | 5.3010 | 6.0370 | 0.0000 | 4.5079 |
| | MathM. | 8.3549 | 6.2852 | 5.7482 | 8.0072 | 5.5387 | 9.6940 | 6.9897 | 7.1844 | 4.5026 | 3.3839 |
| | E-M | -2.5023 | -0.8512 | 9.1962 | 3.2049 | -2.8244 | -0.0111 | -1.6887 | -1.1474 | -4.5026 | 1.124 |
| 5α-androstane-17β-ol & 5β-androstane -3α,17β-diol | Exp. | 16.1778 | 7.0800 | 11.6000 | 16.0400 | 2.5532 | 10.9057 | 7.2500 | 10.3721 | 0.0000 | 5.8333 |
| | MathM. | 17.6358 | 9.1133 | 6.3593 | 12.5177 | 4.6293 | 10.9451 | 7.7706 | 11.9674 | 2.5576 | 4.3175 |
| | E-M | -1.458 | -2.0333 | 5.2407 | 3.5223 | -2.0761 | -0.0394 | -0.5206 | -1.5953 | -2.5576 | 1.5158 |
| 5α-androstane-17β-ol & 5β-androstane -3β,17β-diol | Exp. | 6.6747 | 5.6078 | 13.4054 | 10.1875 | 2.3404 | 5.5200 | 5.8065 | 10.2791 | 0.0000 | 4.3226 |
| | MathM. | 8.7378 | 5.6239 | 5.4412 | 8.6127 | 4.4771 | 5.4223 | 7.4977 | 10.6272 | 3.9262 | 3.7798 |
| | E-M | -2.0631 | -0.0161 | 7.9642 | 1.5748 | -2.1367 | 0.0977 | -1.6912 | -0.3481 | -3.9262 | 0.5428 |
| 5β-androstane-3α,17β -diol & 5β-androstane -3β,17β-diol | Exp. | 1.9333 | 1.0794 | 0.6842 | 2.6786 | 0.2000 | 2.0500 | 2.9903 | 0.0909 | 0.0000 | 0.8529 |
| | MathM. | 2.0475 | 1.7197 | 0.8932 | 1.3022 | 0.4973 | 2.2265 | 2.7229 | 0.8321 | 0.0000 | 0.4648 |
| | E-M | -0.1142 | -0.6403 | -0.209 | 1.3764 | -0.2973 | -0.1765 | 0.2674 | -0.7412 | 0 | 0.3881 |

Exp. = experimental resolution of separation; MathM. = resolution of separation obtained by mathematical model
E-M = Exp - MathM

**Table 7. Results of correlation between experimental and optimized values for separation resolution**

| Parameters | r | 95% $CI_r$ | $r^2$ |
|---|---|---|---|
| 5α-androstane-3β-ol & 5α-androstane-3α-ol | 0.9365 | [0.7477 – 0.9851] | 0.8770 |
| 5α-androstane-3β-ol & 5α-androstane-17β-ol | 0.8602 | [0.5029 – 0.9663] | 0.7400 |
| 5α-androstane-3β-ol & 5β-androstane-3α,17β-diol | 0.7262 | [0.1778 – 0.9303] | 0.5274 |
| 5α-androstane-3β-ol & 5β-androstane-3β,17β-diol | 0.3879 | [0.3198 – 0.8177] | 0.1505 |
| 5α-androstane-3α-ol & 5α-androstane-17β-ol | 0.9632 | [0.8475 – 0.9915] | 0.9277 |
| 5α-androstane-3α-ol & 5β-androstane-3α,17β-diol | 0.7021 | [0.1298 – 0.9234] | 0.4929 |
| 5α-androstane-3α-ol & 5β-androstane-3β,17β-diol | 0.4362 | [0.2666 – 0.8361] | 0.1903 |
| 5α-androstane-17β-ol & 5β-androstane-3α,17β-diol | 0.8682 | [0.5261 – 0.9684] | 0.7538 |
| 5α-androstane-17β-ol & 5β-androstane-3β,17β-diol | 0.5850 | [0.0706 – 0.8876] | 0.3423 |
| 5β-androstane-3α,17β-diol & 5β-androstane-3β,17β-diol | 0.8346 | [0.4319 – 0.9598] | 0.6966 |

r = correlation coefficient; 95% $CI_r$ = 95% confidence intervals for correlation coefficient;
$r^2$ = squared correlation coefficient

**Discussion**

The proposed mathematical model for optimization of mobile phase of androstane isomers has been developed, the aim of the research being reached.

As it can be observed from the Results chapter three parameters were optimized: the retention factor, the separation resolution and the objective function. Analyzing the obtained results (Table 3) two observation can be done. First observation refers the generic dependence equation used: for all three parameters the best results were obtained with the seven parameters equation (Eq.(2)). The second observation refers the optimum mobile phase which is identical for separation resolution and objective function (Chloroform : Cyclohexan : Methyl-Ethyl-Cetone = 64 : 0 : 36, see Table 3), and are very closed to the optimum mobile phase obtained for the retention factor that is of 60 : 13 : 27.

Analyzing the data from Table 4 it can be observed that the differences between retention factor obtained from experimental data and the value obtained through optimization were very small, this sustaining the abilities of the optimization procedure. The minimum value of the absolute difference between these values is of 0.0000 (see the results from the Table 4, the experiment number 9) to 0.2390 (see the results from experiment number 4, for the 5α-androstane-3α-ol isomer. Note that the value obtained through optimization was less than the value obtained from experimental data (see Table 4).

The performances of the proposed optimization model can be analyzed thought the correlation coefficient and associated squared value. The closer the value of correlation coefficient is to -1 or +1 the better the optimization procedure is. Looking at the correlation coefficients presented in Table 5 it can be observed that in four cases out of five the value is greater than 0.99. The exception is observed for the compound 5α-androstane 3α-ol for each the correlation coefficient is of 0.9648, being considered that there is a strong relationship between experimental value and value obtained through optimization procedure.

The performances obtained by using of the proposed mathematical model for the separation resolution parameter are not so good comparing with those obtained for retention factor (see Table 6). This difference can be explained by the difference between the distributions of data: the differences between the maximum and minimum value obtained from experimental data vary from 0.0000 to 0.6825 for the retention factor (experiment number 6), and from 0.0000 to 21.9636 for the resolution of separation. The differences between experimental and optimized value differ from one experiment to other. For example, for the first experiment with one exception (separation resolution between 5α-androstane-3α-ol and 5α-androstane-17β-ol), the value obtained though optimization vas greater comparing with value obtained experimentally. The greater difference in separation resolution of 2.5023 was observed for 5α-androstane-3α-ol and 5β-androstane-3β,17β-diol compounds (first experiment, Table 6). At the opposite sides are experiment no. three and ten. In those experiments, with one exception the value obtained through optimization was smaller comparing with the value obtained experimentally. The greatest difference was of 9.1962 for the experiment number 3, resolution separation between 5α-androstane-3α-ol and 5β-androstane-3β,17β-diol, and of 1.5645 for experiment number ten (separation resolution between 5α-androstane-3α-ol and 5β-androstane-3α,17β-diol). Analyzing the performances of the optimization procedure, regarding the resolution separation, the best performances were obtained for 5α-androstane-3α-ol and 5α-androstane-17β-ol compounds (r = 0.9632, see Table 7). Thus, for the separation resolution the mathematical model had not the same stability as for the retention factor (the value of the correlation coefficients vary from 0.3879 to 0.9632, with two values less than 0.5, three values between 0.5-0.75, and five values greater than 0.75).

Some performance was obtained in optimization of objective function, but this performance is less comparing with the performances obtained for optimization of the retention factor.

The assessment of the proposed mathematical model in optimization of the mobile phase of the androstane isomers by thin layer chromatography with three solvents can be done by analyzing the obtained results and of advantages and disadvantages. The greatest advantage of the mathematical model results from its faster ability in obtaining the optimum composition of the mobile phase. Looking at the obtained composition of the optimum mobile phase it can be observed that the optimization of

the retention factor, of the separation resolution and of the objective function lead to very similar compositions of mobile phases (see Table 3). This observation can be explained by the moderately polar character of the androstane isomers and shown us that the small variations in composition of the optimum mobile phase do not lead to significant modification of compounds separation.

As a concluding remark it can be say that the proposed mathematical model proved to assure accurate results on analysis of the separation of androstane isomers. But, more researches are necessary to be done in order to analyze the stability and validity of the proposed model. If the mathematical model will prove its stability and validity could become a useful method in separation of androstane isomers from drugs, biological or natural sources.

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