3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

1. OBJECTIVE

Present the results obtained in the validation of the analytical method for assay of Keto acids and Hydroxy acids in Ketosteril drug-coated tablets, by HPLC using UV detection.

2. DEFINITIONS AND ABBREVIATIONS

RSD – Relative Standard Deviation
rt – Retention time
Resol. – Resolution

3. RESULTS

3.1. FORMULA DESCRIPTION

3.1.1. Active Substance

Amino acids, keto acids and hydroxyl acids described in the formulation (point 3.1.3)

3.1.2. Pharmaceutical form

Coated tablets

3.1.3. Formulation

- Composition of the cores

Components	Amount per unit (mg)	Function
Alfa-Ketoisoleucine, calcium**	67,0**	Active ingredient
Alfa-Ketoleucine, calcium**	101,0**	Active ingredient
Alfa-Ketophenylalanine, calcium**	68,0**	Active ingredient
Alfa-Ketovaline, calcium**	86,0**	Active ingredient
Alfa-Hydroxymethionine, calcium**	59,0**	Active ingredient
Isopropyl alcohol*	101,666 – 108,333 0,1295 – 0,138 ml	Solvent
Purified water*	0,001666 – 0,005	Solvent
Povidone	26,3	Granulating agent
Macrogol 6000	7,8	Lubricant
L-Lysine acetate	105,0	Active ingredient
L-Threonine	53,0	Active ingredient
L-Tryptophan	23,0	Active ingredient
L-Histidine	38,0	Active ingredient
L-Tyrosine	30,0	Active ingredient

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Components	Amount per unit (mg)	Function
Isopropyl alcohol*	0,0375 0,0478 ml	Solvent
Purified water*	0,0015	Solvent
Povidone	16,883	Granulating agent
Macrogol 6000	5,0	Lubricant
Maize starch***	Max. 44	Filler, balancing agent
Talc	19,6	Release agent
Silica colloidal anhydrous	4,40	Flow regulator
Crospovidone	6,00	Explosive
Magnesium stearate	15,0	Lubricant
Total		**≈ 775**

* eliminated during the process;
** the individual water-content is taken into account in order to achieve 100% active ingredient content in each batch;
***varies, as used as filling agent in order to achieve a constant tablet core weight with specified continuous active ingredient content.

- Composition of the film-coat

Components	Amount per unit (mg)	Function
Isopropyl alcohol*	70 89,166 ml	Solvent
Talc	9,067	Release agent
Titanium dioxide	7,40	Pigment
Macrogol 6000	1,2	Lubricant
Purified water*	1,2	Solvent
Quinoline yellow E 104	0,72	Pigment
Purified water*	6	Solvent
Eudragit E 12,5% m/m**	47,2 (5,9 solid)	Coating compound
Triacetin	0,43	Plasticizer
Macrogol 6000	0,28	Lubricant
Purified water*	0,22	Solvent
Isopropyl alcohol*	2,017 0,0025688 ml	Solvent
Total		**≈ 25**

* eliminated during the process;
** part eliminated during the process.

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

3.2. METHOD

3.2.1. Reagents and materials

0.5M Sulfuric acid (VWR, batch: 15I300515, validity: 30 Sep. 2017)
1N Sulfuric acid (logbook RU2, 49/2016, page 126)
MilliQ water, obtained from a Millipore System
Alfa-ketoisoleucine calcium primary standard (Evonik, batch: 3427550206, validity: 09 June 2017, activity: 89.6%)
Alfa-ketoleucine calcium primary standard (Evonik, batch: 2427050102, validity: 09 June 2017, activity: 89.8%)
Alfa-ketophenylalanine calcium primary standard (Evonik, batch: 2428450130, validity: 09 June 2017, activity: 93.9%)
Alfa-ketovaline calcium primary standard (Evonik, batch: 3428750231, validity: 09 June 2017, activity: 91.2%)
Alfa-Hydroxymethionine calcium primary standard (Evonik, batch: 2427950205, validity: 09 June 2017, activity: 99.2%)
Povidone (Batch: 160300498, validity: 30 June 2018)
Macrogol 6000 (Batch: 160100494, validity: 16 July 2019)
L-Lysine acetate (Batch: 160500195, validity: 02 Feb. 2019)
L-Threonine (Batch: 160400321, validity: 11 Mar. 2022)
L-Tryptophan (Batch: 160300029, validity: 26 Feb. 2018)
L-Histidine (Batch: 160400068, validity: 17 Nov. 2018)
L-Tyrosine (Batch: 160300620, validity: 29 Feb. 2020)
Maize Starch (Batch: 150500002, validity: 28 Feb. 2017)
Talc (Batch: 160100099, validity: 18 June 2018)
Silica colloidal anhydrous (Batch: 160100499, validity: 21 Oct. 2017)
Crospovidone (Batch: 160100468, validity: 28 Oct. 2018)
Magnesium stearate (Batch: 160100057, validity: 31 Oct. 2017)
Titanium dioxide (Batch: 150800228, validity: 07 July 2018)
Quinoline yellow E 104 (Batch: 150900495, validity: 23 July 2020)
Eudragit E 12,5% (Batch: 151100190, validity: 31 Jan. 2018)
Triacetin (Batch: 150400580, validity: 30 Sep. 2019)
Ketosteil tablets (Batch: 18N1032, validity: Mar. 2019)
Volumetric flasks, micropipettes and pipettes

3.2.2. Equipment

5120-II-0286: HPLC with quaternary pump, autosampler, Diode Array UV/UV-Vis detector and software of acquisition and treatment data
5120-II-0417: Waters, UPLC with Functionality of HPLC, equipped with two binary pumps, autosampler, Diode Array UV/UV-Vis detector and software of acquisition and treatment data

3.2.3. Standard stock solution: High range linearity - analytes reference standards

It was weighed 212.2 mg of alfa-ketoisoleucine, 311.9 mg of alfa-ketoleucine, 263.1 mg of alfa-ketovaline, 191.9 mg of alfa-ketophenylalanine and 151.3 mg of alfa-hydroxymethionine in to a 1000 mL volumetric flask. 40 mL of 1N sulfuric acid were added and the volume was filled up with water.

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

3.2.4. Standard working solution: High range linearity - analytes reference standards

Standard working solutions were prepared according to table 1.

Level (%)	Dilution (mL standard stock solution → mL)
50	5 → 100
80	8 → 100
90	9 → 100
100	10 → 100
110	11 → 100
120	12 → 100
150	15 → 100

Table 1

To each 100 mL volumetric flask was added 3.6 mL of 1N acid sulfuric and then filled up with water.

3.2.5. Standard stock solution: High range linearity - matrix effect

It was weighed 212.2 mg of alfa-ketoisoleucine, 311.9 mg of alfa-ketoleucine, 263.4 mg of alfa-ketovaline, 191.8 mg of alfa-ketophenylalanine and 151.1 mg of alfa-hydroxymethionine in to a 100 mL volumetric flask. 4 mL of 1N sulfuric acid were added and the volume was filled up with water.

3.2.6. Standard working solution: High range linearity - matrix effect

Standard working solutions were prepared according to table 2.

Level (%)	Dilution (mL standard stock solution → mL)
50	5 → 1000
80	8 → 1000
90	9 → 1000
100	10 → 1000
110	11 → 1000
120	12 → 1000
150	15 → 1000

Table 2

To each 1000 mL volumetric flask was added placebo amounts corresponding to the 100% composition and 36 mL of 1N sulfuric acid. The volume was filled up with water.

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

3.2.7. Standard solution

It was weighed about 212.1 mg of alfa-ketoisoleucine, 311.8 mg of alfa-ketoleucine, 263.2 mg of alfa-ketovaline, 191.7 mg of alfa-ketophenylalanine and 151.2 mg of alfa-hydroxymethionine in to a 1000 mL volumetric flask. 40mL of 1N sulfuric acid were added and the volume was filled up with water. Finally, 10 mL of the previous solution were mixed with 3.6 mL of 1N sulfuric acid and water was added to provide 100 mL solution.

3.2.8. Test solution

It was weighed 10 tablets of Ketosteril and transferred to a 2000 mL volumetric flask. 1800 mL of water and 80 mL of 1N sulfuric acid were added and the solution was stirred until tablets complete dissolution. Then the volume was filled up with water.
Finally, 5 mL of solution were mixed with 3.8 mL of 1N sulfuric acid and water was added to provide 100mL solution.

3.2.9. Chromatographic conditions

- Column: Aminex HPX-87H, 300x7.8 mm, Catalog number: 125-0140, Serial number: 444205, Internal number: 0164/16
- Column: Aminex HPX-87H, 300x7.8 mm, Catalog number: 125-0140, Serial number: 445098, Internal number: 0162/16 (Robustness- variation of column batch)
- λ= 205 nm
- Mobile phase: 0.025M sulfuric acid – Mixture of 50 mL of 0.5M sulfuric acid with 950 mL of water.
- Mobile phase: 0.024M sulfuric acid – Mixture of 48 mL of 0.5M sulfuric acid with 952 mL of water (Robustness- variation of mobile phase composition)
- Mobile phase: 0.026M sulfuric acid – Mixture of 52 mL of 0.5M sulfuric acid with 948 mL of water (Robustness- variation of mobile phase composition)
- Flow rate: 0.45 mL/min
- Flow rate: 0.35 mL/min and 0.55 mL/min (Robustness - variation of the flow rate)
- Column temperature: Room temperature
- Column temperature: 15ºC and 25ºC (Robustness - variation of the column temperature)
- Sample tray temperature: 5ºC
- Injection volume: 50 µL

3.3. VALIDATION PARAMETERS

3.3.1. System Suitability

Acceptance criteria:
- The resolution between all the 5 peaks should not be less than 1
- The RSD between 5 injections of the standard solution should not be more than 3.66%

Substance	Resolution	RSD (%)
Alfa-Ketovaline	6.504	0.35
Alfa-Ketoisoleucine	3.233	0.30
Alfa-Ketoleucine	1.649	0.18
Alfa-Hydroxymethionine	3.362	0.45

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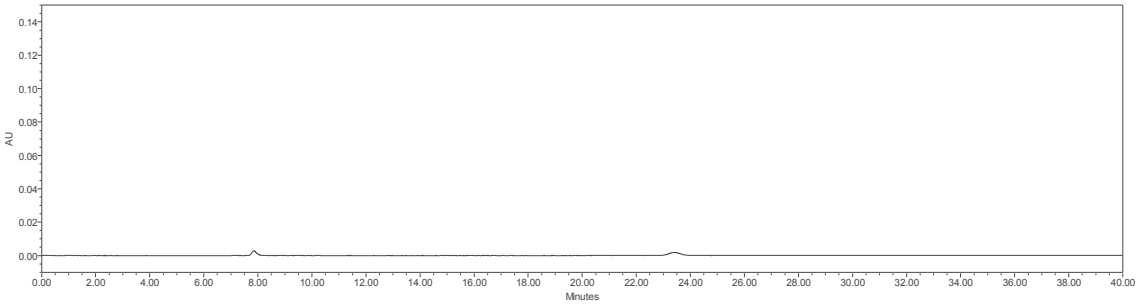
Substance	Resolution	RSD (%)
Alfa-Ketophenylalanine	3.362	0.52

3.3.2. Selectivity

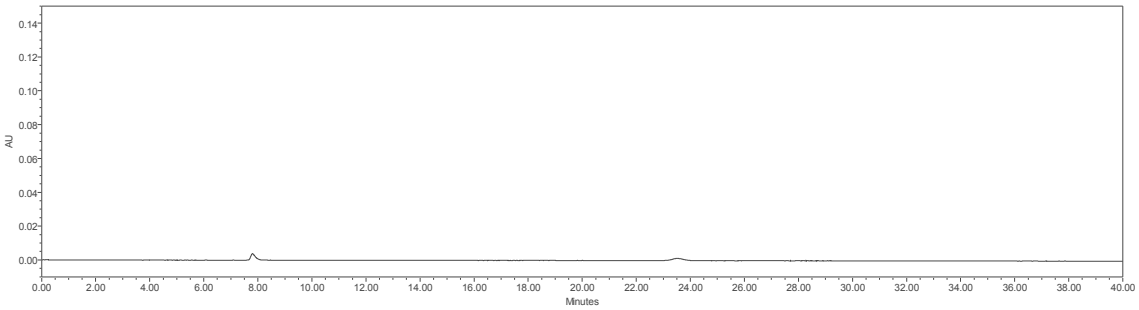
The placebo solution doesn't interfere in the Keto and Hydroxy acids quantification.

3.3.2.1. Mobile Phase



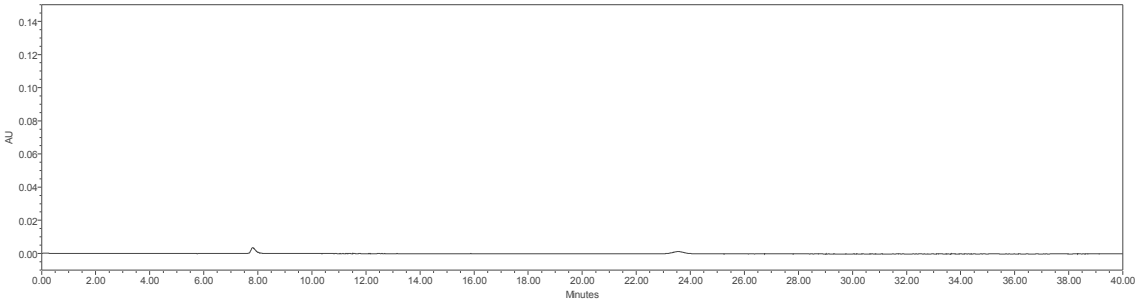
Chromatogram 1

3.3.2.2. Standard Blank



Chromatogram 2

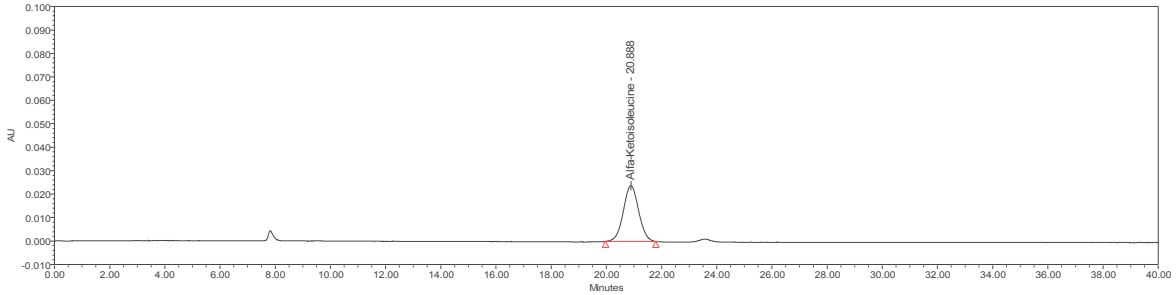
3.3.2.3. Sample Blank



Chromatogram 3

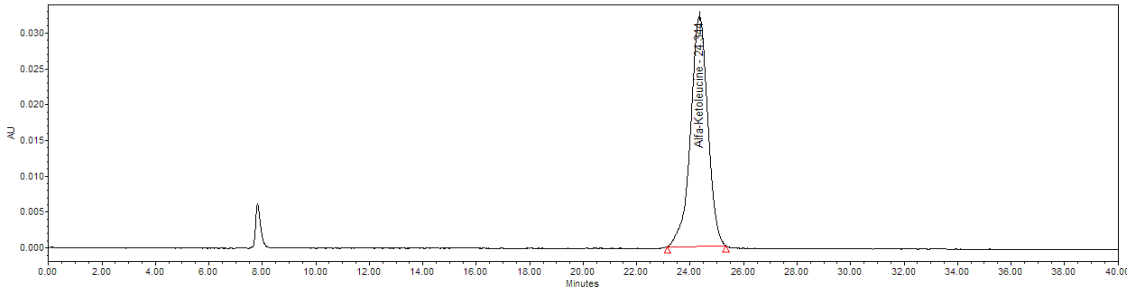
3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

3.3.2.4. Alfa-Ketoisoleucine standard solution



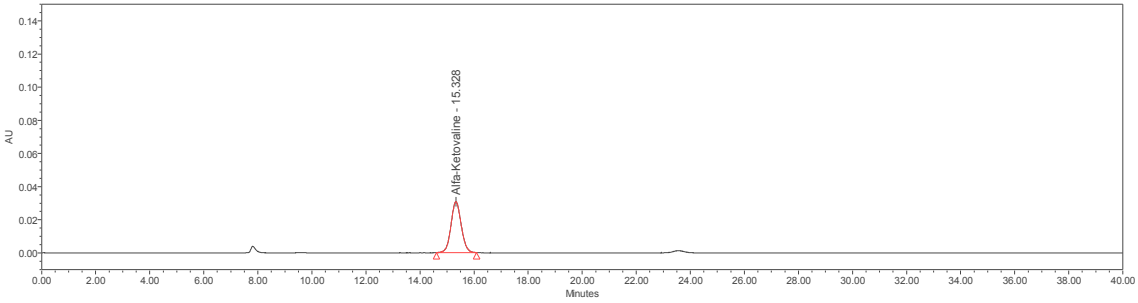
Chromatogram 4

3.3.2.5. Alfa-Ketoleucine standard solution



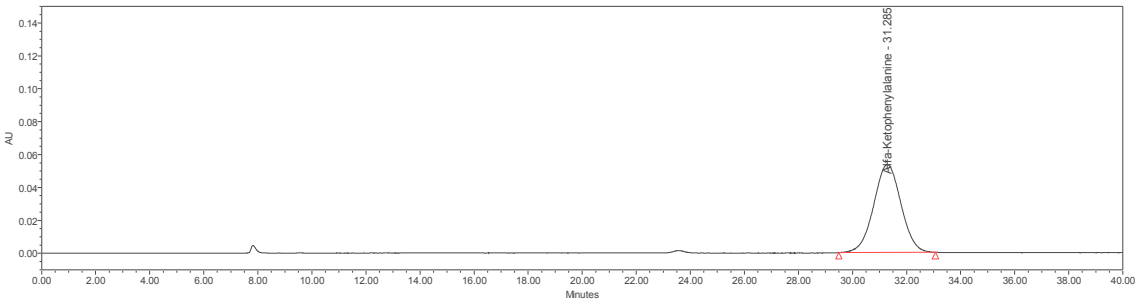
Chromatogram 5

3.3.2.6. Alfa-Ketovaline standard solution



Chromatogram 6

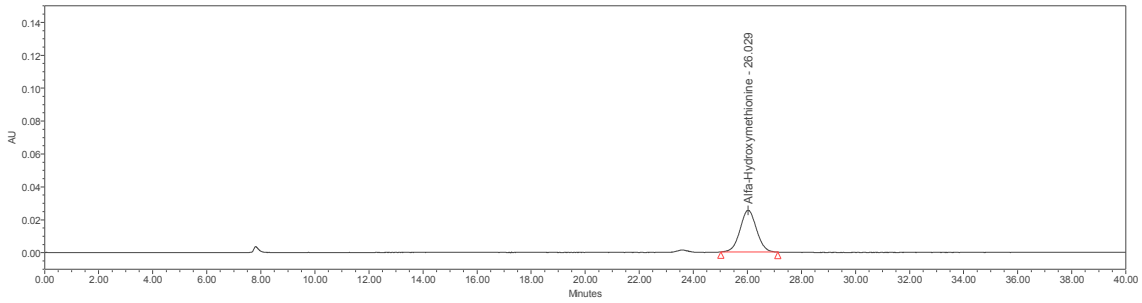
3.3.2.7. Alfa-Ketophenylalanine standard solution



Chromatogram 7

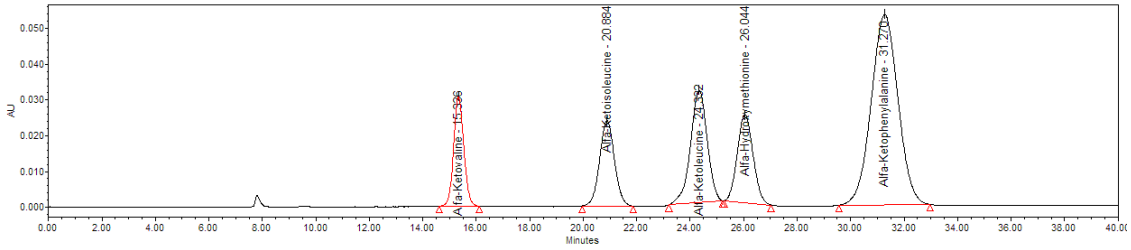
3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

3.3.2.8. Alfa-Hydroxymethionine standard solution



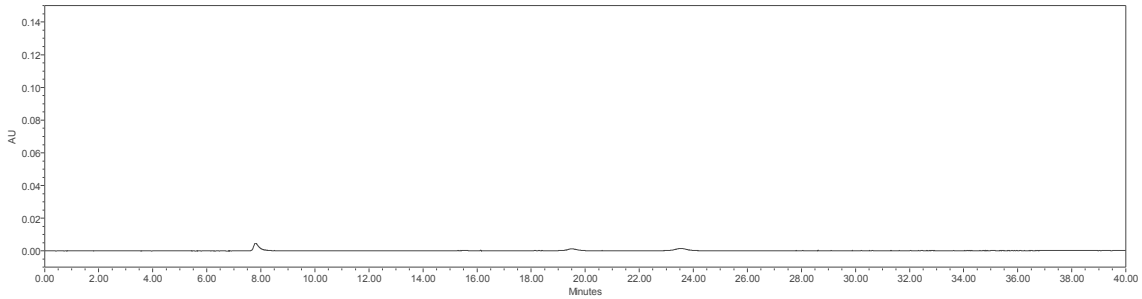
Chromatogram 8

3.3.2.9. Standard solution



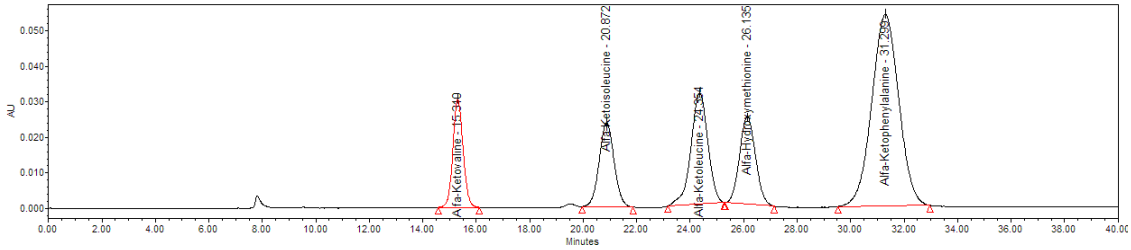
Chromatogram 9

3.3.2.10. Placebo solution



Chromatogram 10

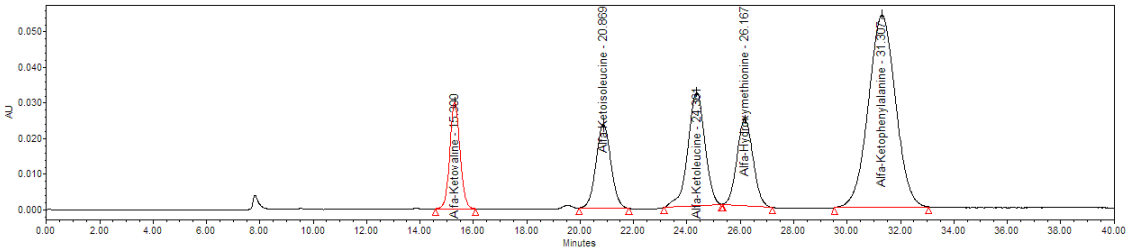
3.3.2.11. Placebo + standard solution



Chromatogram 11

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

3.3.2.12. Test solution



Chromatogram 12

3.3.3. High range linearity

a) Analytes reference standards

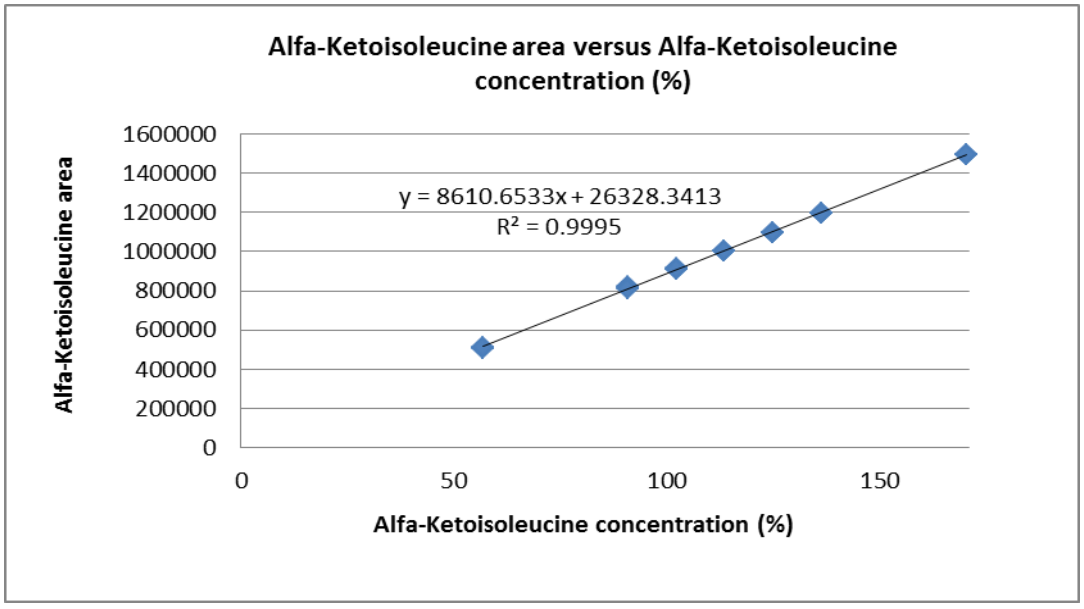
Acceptance criteria:
- $r \geq 0{,}990$
- The residuals must have a random distribution and the 95% or 99% confidence interval for the intercept term must contain zero.

- Alfa-Ketoisoleucine

Level (%)	Alfa-Ketoisoleucine concentration (%)	Area
50	56.756	506881
		509086
		510014
	Mean	508661
	RSD (%)	0.3
80	90.809	818635
		822732
		810591
	Mean	817320
	RSD (%)	0.8
90	102.160	915081
		912621
		911971
	Mean	913224
	RSD (%)	0.2
100	113.511	1003415
		1005376
		1000914
	Mean	1003235
	RSD (%)	0.2

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Level (%)	Alfa-Ketoisoleucine concentration (%)	Area
110	124.862	1095870
		1096832
		1094636
	Mean	1095779
	RSD (%)	0.1
120	136.213	1192882
		1193868
		1193018
	Mean	1193256
	RSD (%)	0.0
150	170.267	1496835
		1494578
		1492570
	Mean	1494661
	RSD (%)	0.1



Linear regression equation (y=8610.6533x + 26328.3413)

m= 8610.6533
b= 26328.3413
r= 0.9998

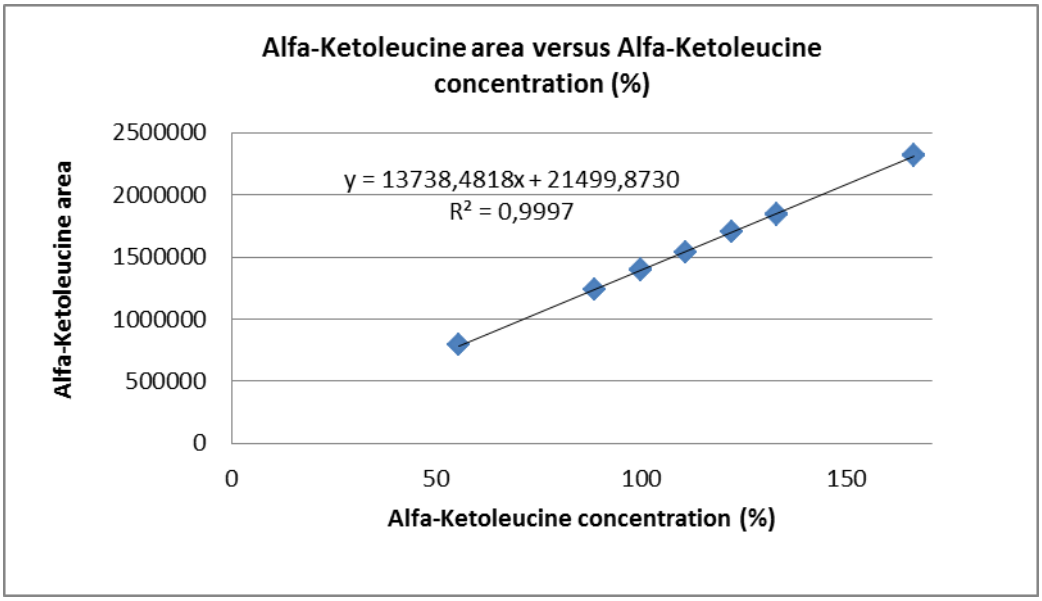
3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

- Alfa-Ketoleucine

Level (%)	Alfa-Ketoleucine concentration (%)	Area
50	55.463	793686
		791743
		793329
	Mean	792919
	RSD (%)	0.1
80	88.740	1237791
		1239345
		1232216
	Mean	1236451
	RSD (%)	0.3
90	99.833	1390337
		1389464
		1399831
	Mean	1393211
	RSD (%)	0.4
100	110.925	1534306
		1532938
		1531932
	Mean	1533058
	RSD (%)	0.1
110	122.018	1697519
		1701470
		1702399
	Mean	1700463
	RSD (%)	0.2
120	133.110	1847734
		1839855
		1848533
	Mean	1845374
	RSD (%)	0.3
150	166.388	2318182
		2316825
		2314891

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Level (%)	Alfa-Ketoleucine concentration (%)	Area
	Mean	2316633
	RSD (%)	0.1



Linear regression equation (y=13738.4818x + 21499.8730)

m= 13738.4818
b= 21499.8730
r= 0.9998

- Alfa-Ketovaline

Level (%)	Alfa-Ketovaline concentration (%)	Area
50	55.802	441234
		442690
		442597
	Mean	442173
	RSD (%)	0.2
80	89.283	709815
		709070
		704559
	Mean	707815
	RSD (%)	0.4
90	100.443	799423
		792816

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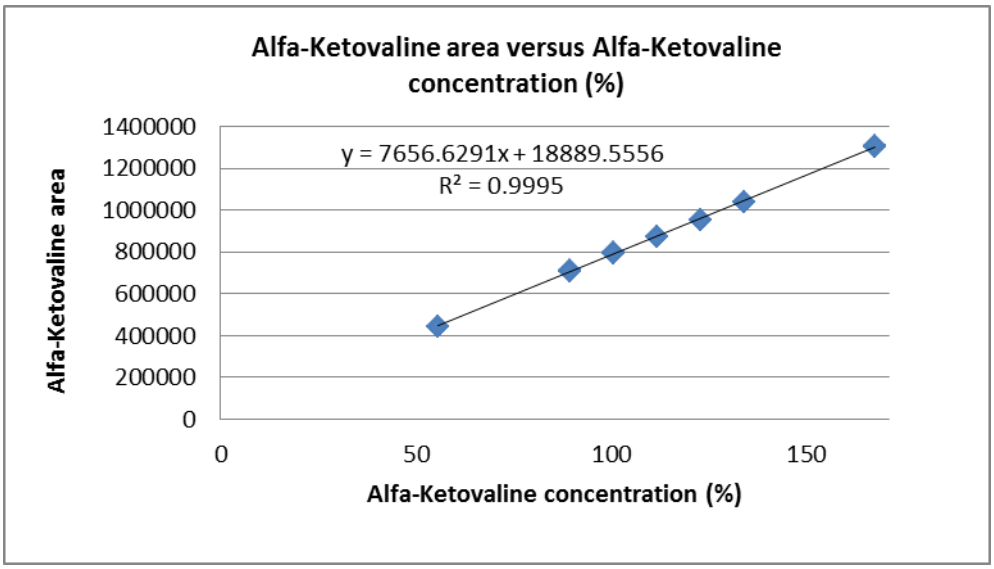
Level (%)	Alfa-Ketovaline concentration (%)	Area
		793859
	Mean	795366
	RSD (%)	0.5
100	111.603	873996
		875076
		871464
	Mean	873512
	RSD (%)	0.2
110	122.764	954930
		952540
		951665
	Mean	953045
	RSD (%)	0.2
120	133.924	1039801
		1038051
		1035565
	Mean	1037805
	RSD (%)	0.2
150	167.405	1301111
		1304427
		1306606
	Mean	1304048
	RSD (%)	0.2

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)



Linear regression equation (y=7656.6291x + 18889.5556)

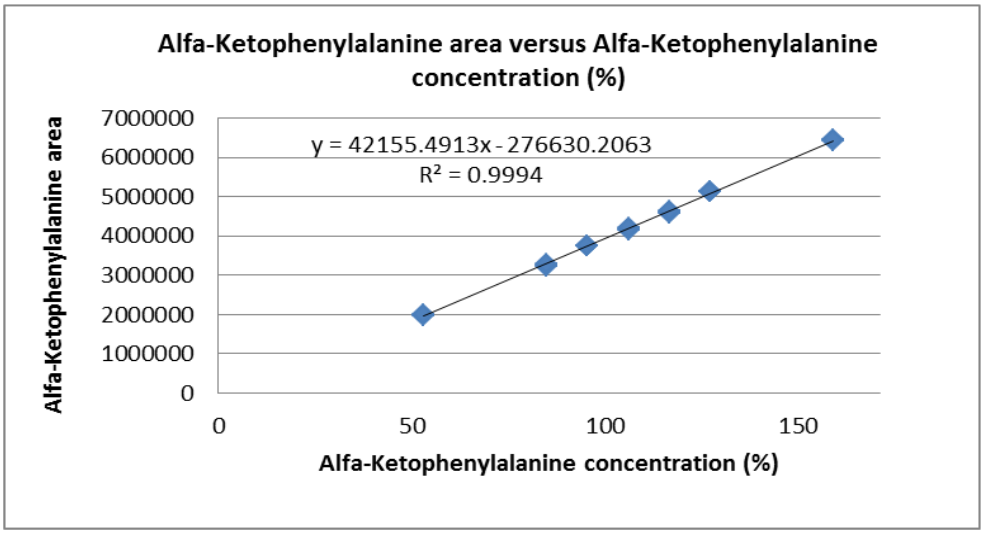
m= 7656.6291
b= 18889.5556
r= 0.9998

- Alfa-Ketophenylalanine

Level (%)	Alfa-Ketophenylalanine concentration (%)	Area
50	52.998	1979803
		1987057
		2001850
	Mean	1989570
	RSD (%)	0.6
80	84.797	3236269
		3251087
		3279540
	Mean	3255632
	RSD (%)	0.7
90	95.397	3741207
		3743493
		3759509
	Mean	3748069
	RSD (%)	0.3
100	105.997	4178936

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Level (%)	Alfa-Ketophenylalanine concentration (%)	Area
		4154941
		4192039
	Mean	4175305
	RSD (%)	0.5
110	116.596	4610528
		4590835
		4649039
	Mean	4616801
	RSD (%)	0.6
120	127.196	5120988
		5134189
		5127232
	Mean	5127469
	RSD (%)	0.1
150	158.995	6420304
		6433497
		6433474
	Mean (%)	6429092
	RSD (%)	0.1



Linear regression equation (y=42155.4913x – 276630.2063)

m= 42155.4913
b= -276630.2063
r= 0.9997

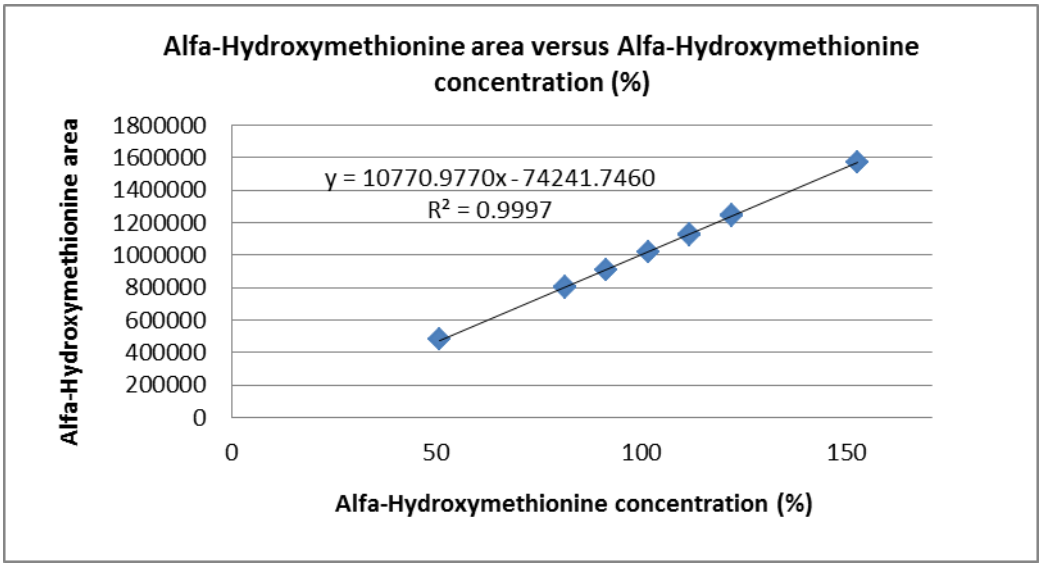
3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

- Alfa-Hydroxymethionine

Level (%)	Alfa-Hydroxymethionine concentration (%)	Area
50	50.878	480248
		481143
		479957
	Mean	480450
	RSD (%)	0.1
80	81.405	800906
		803026
		801830
	Mean	801921
	RSD (%)	0.1
90	91.580	905505
		905278
		907994
	Mean	906259
	RSD (%)	0.2
100	101.756	1018089
		1016289
		1015708
	Mean	1016695
	RSD (%)	0.1
110	111.931	1129187
		1124771
		1126862
	Mean	1126940
	RSD (%)	0.2
120	122.107	1249451
		1246906
		1242682
	Mean	1246346
	RSD (%)	0.3
150	152.633	1573928
		1573523

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Level (%)	Alfa-Hydroxymethionine concentration (%)	Area
		1573806
	Mean	1573753
	RSD (%)	0.0



Linear regression equation (y=10770.9770x – 74241.7460)

m= 10770.9770
b= -74241.7460
r= 0.9999

b) Matrix effect: placebo + analytes reference standards
Acceptance criteria:
- r ≥ 0,990
- The residuals must have a random distribution and the 95% or 99% confidence interval for the intercept term must contain zero.

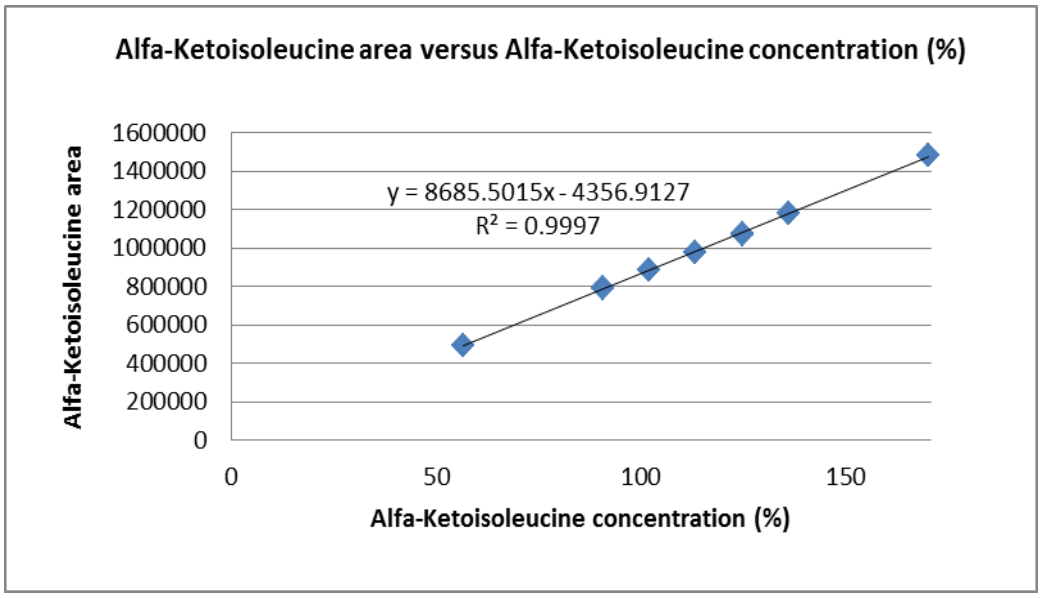
- Alfa-Ketoisoleucine

Level (%)	Alfa-Ketoisoleucine concentration (%)	Area
50	56.756	491930
		491000
		488151
	Mean	490360
	RSD (%)	0.4
80	90.809	793275
		791397

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Level (%)	Alfa-Ketoisoleucine concentration (%)	Area
		785366
	Mean	790013
	RSD (%)	0.5
90	102.160	881303
		885139
		883118
	Mean	883187
	RSD (%)	0.2
100	113.511	977905
		978815
		974957
	Mean	977226
	RSD (%)	0.2
110	124.862	1074092
		1068744
		1071391
	Mean	1071409
	RSD (%)	0.3
120	136.213	1176361
		1178186
		1179525
	Mean	1178024
	RSD (%)	0.1
150	170.267	1478466
		1480248
		1483065
	Mean	1480593
	RSD (%)	0.2

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)



Linear regression equation (y=8685.5015x – 4356.9127)

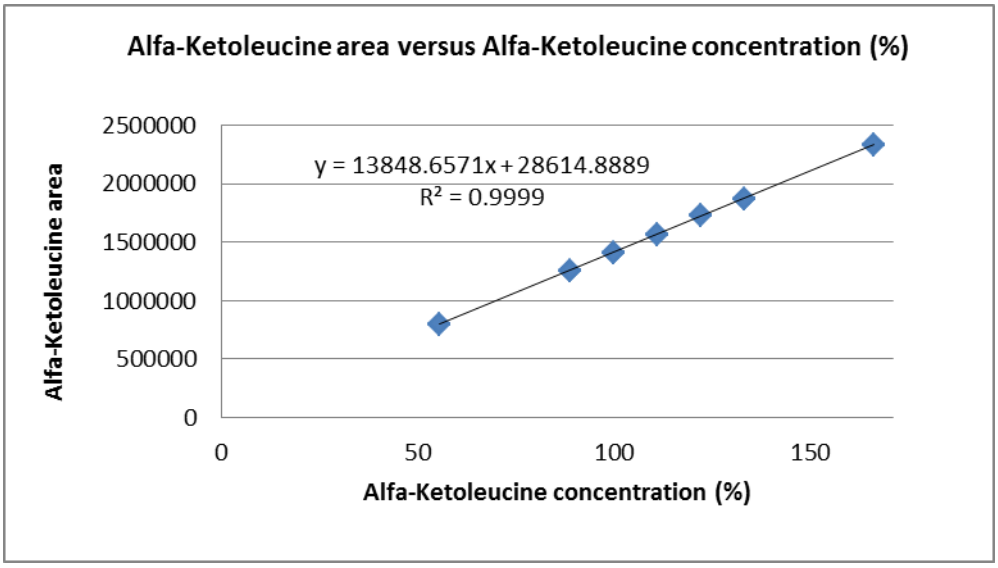
m= 8685.5015
b= -4356.9127
r= 0.9998

- Alfa-Ketoleucine

Level (%)	Alfa-Ketoleucine concentration (%)	Area
50	55.463	797671
		794044
		798243
	Mean	796653
	RSD (%)	0.3
80	88.740	1254123
		1256986
		1254317
	Mean	1255142
	RSD (%)	0.1
90	99.833	1410659
		1406038
		1409261
	Mean	1408652
	RSD (%)	0.2
100	110.925	1561419

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Level (%)	Alfa-Ketoleucine concentration (%)	Area
		1569346
		1569588
	Mean	1566784
	RSD (%)	0.3
110	122.018	1717169
		1728040
		1728011
	Mean	1724406
	RSD (%)	0.4
120	133.110	1878862
		1868882
		1866590
	Mean	1871445
	RSD (%)	0.4
150	166.388	2336088
		2322742
		2332308
	Mean	2330380
	RSD (%)	0.3



Linear regression equation (y=13848.6571x + 28614.8889)

m= 13848.6571
b= 28614.8889

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

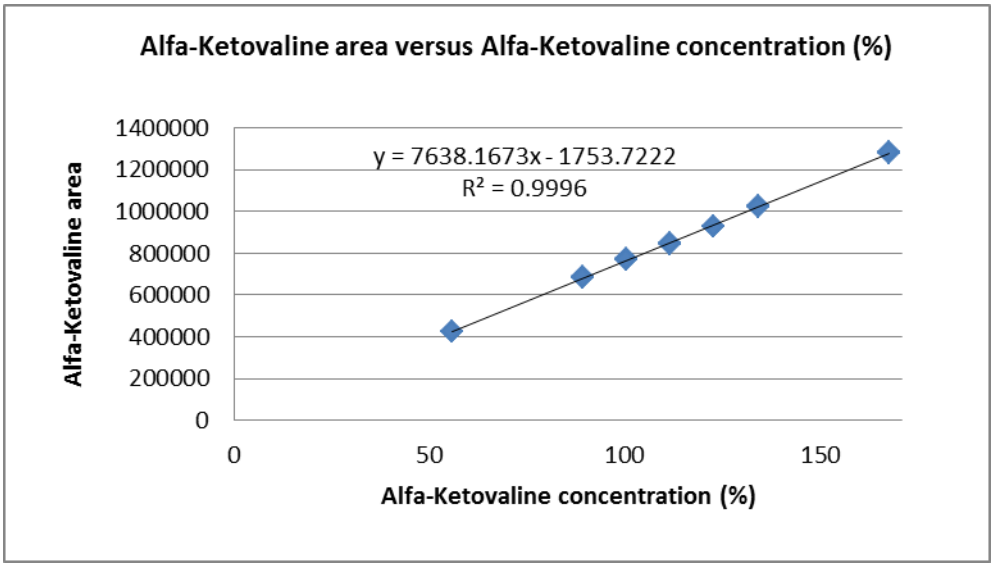
r= 0.9999

- Alfa-Ketovaline

Level (%)	Alfa-Ketovaline concentration (%)	Area
50	55.865	426281
		426857
		424926
	Mean	426021
	RSD (%)	0.2
80	89.384	687510
		684029
		683557
	Mean	685032
	RSD (%)	0.3
90	100.558	772658
		767491
		766610
	Mean	768920
	RSD (%)	0.4
100	111.731	849507
		844420
		845980
	Mean	846636
	RSD (%)	0.3
110	122.904	929747
		930888
		925071
	Mean	928569
	RSD (%)	0.3
120	134.077	1023461
		1025236
		1022609
	Mean	1023769
	RSD (%)	0.1
150	167.596	1278288

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Level (%)	Alfa-Ketovaline concentration (%)	Area
		1285969
		1283835
	Mean	1282697
	RSD (%)	0.3



Linear regression equation (y=7638.1673x – 1753.7222)

m= 7638.1673
b= -1753.7222
r= 0.9998

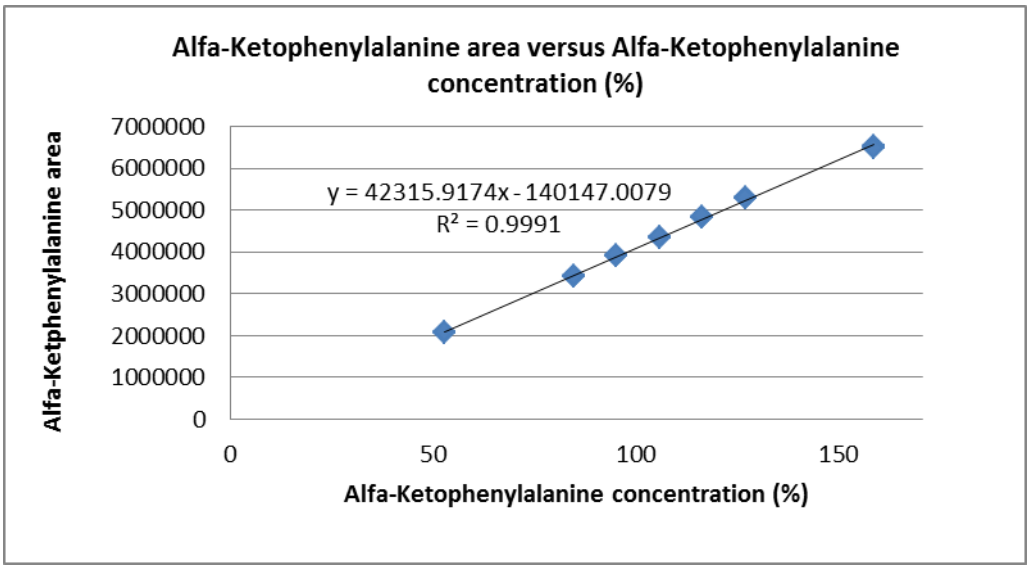
- Alfa-Ketophenylalanine

Level (%)	Alfa-Ketophenylalanine concentration (%)	Area
50	52.971	2058594
		2085593
		2082713
	Mean	2075633
	RSD (%)	0.7
80	84.753	3407244
		3414757
		3429535
	Mean	3417179
	RSD (%)	0.3
90	95.347	3889091

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Level (%)	Alfa-Ketophenylalanine concentration (%)	Area
		3913459
		3922159
	Mean	3908236
	RSD (%)	0.4
100	105.941	4343959
		4362224
		4365262
	Mean	4357148
	RSD (%)	0.3
110	116.535	4825104
		4822749
		4844006
	Mean	4830620
	RSD (%)	0.2
120	127.130	5304283
		5279204
		5284234
	Mean	5289240
	RSD (%)	0.3
150	158.912	6552149
		6523121
		6490537
	Mean	6521935
	RSD (%)	0.5

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)



Linear regression equation (y=42315.9174x – 140147.0079)

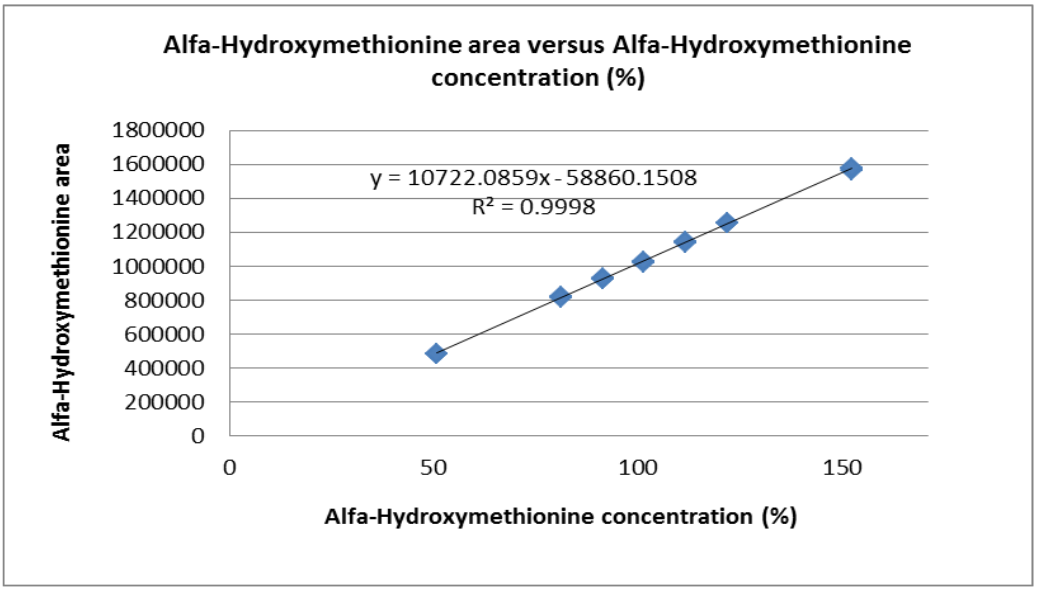
m= 42315.9174
b= -140147.0079
r= 0.9995

- Alfa-Hydroxymethionine

Level (%)	Alfa-Hydroxymethionine concentration (%)	Area
50	50.811	480852
		481665
		483077
	Mean	481865
	RSD (%)	0.2
80	81.297	817129
		816837
		815100
	Mean	816355
	RSD (%)	0.1
90	91.459	927453
		925593
		924907
	Mean	925984
	RSD (%)	0.1
100	101.621	1020391

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Level (%)	Alfa-Hydroxymethionine concentration (%)	Area
		1025707
		1029098
	Mean	1025065
	RSD (%)	0.4
110	111.783	1137860
		1141255
		1144551
	Mean	1141222
	RSD (%)	0.3
120	121.945	1251360
		1256853
		1250558
	Mean	1252924
	RSD (%)	0.3
150	152.432	1563725
		1576174
		1575197
	Mean	1571699
	RSD (%)	0.4



Linear regression equation (y=10722.0859x – 58860.1508)

m= 10722.0859

b= -58860.1508
r= 0.9999

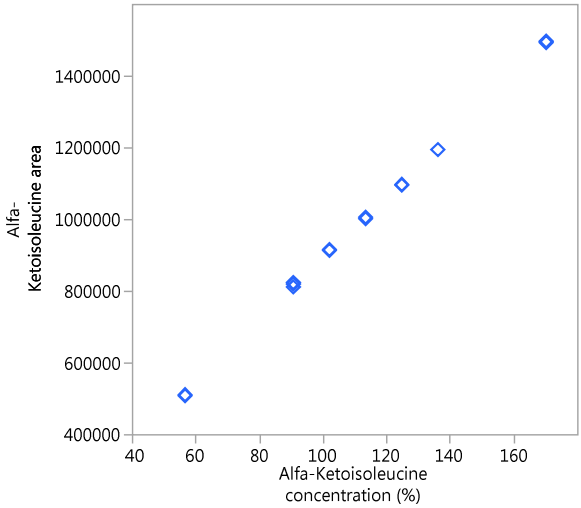
c) Statistical analysis

The statistical analysis was performed with assistance of the statistical software JMP Statistical Discovery from SAS, version 12 and XLSTAT 2016.

Alfa-ketoisoleucine

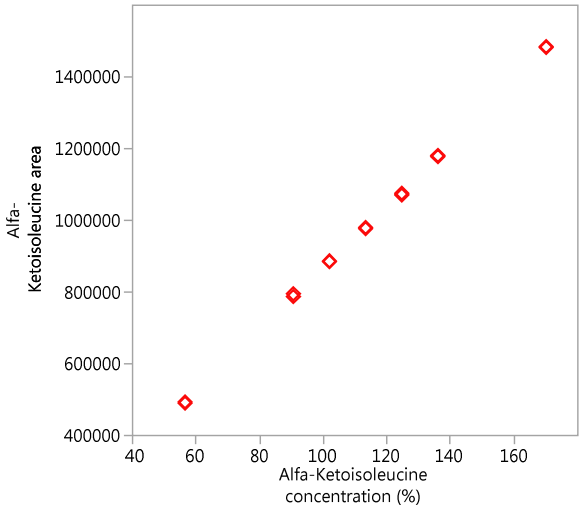
Scatterplots

In order to assess whether there is a linear relationship between the Concentration (%) and the Area of the analyte, two scatterplots were constructed, one in which the analyte is the only component of the sample, and another in which the analyte is in a complex matrix with other constituents.
Three different areas were observed for each of the seven concentrations.
In the case where the analyte is the only component of the sample, the following scatterplot shows a nearly perfect linear relationship.



It is also observed an almost perfect linear relationship between Area and Concentration (%) when the analyte is contained in a complex matrix.

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)



Pearson´s correlation coefficient

Considering the correlation revealed in the two scatterplots, Pearson's correlation coefficients were estimated in order to understand if these were higher than the minimum acceptable, 0.990, which was verified. When the analyte is the only component of the sample, r=0.9998; and when the analyte is contained in a complex matrix, r=0.9998.

Adjusting the linear regression lines using the Least Squares method

After having been verified a linear correlation between the two variables, through a scatterplot and its Pearson's correlation coefficient (r≥0.990), two linear regression lines were estimated, one when the analyte is the only component of the sample,

$$Area = a_1 + b_1 \times Concentration(\%)$$

And another when the analyte is contained in a complex matrix,

$$Area = a_2 + b_2 \times Concentration(\%)$$

Intercepts, a_1 and a_2, and slopes, b_1 and b_2, estimates were obtained from the method of least squares.

In the first case,

	Coefficients	Std. Error	t Stat	P-value	Lower 95%	Upper 95%
Intercept	26328,341	5205,659	5,058	0,000	15432,772	37223,910
Conc. (%)	8610,653	44,013	195,640	0,000	8518,533	8702,773

In the second case,

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-4356,913	4328,321	-1,007	0,327	-13416,192	4702,366
Conc. (%)	8685,502	36,595	237,340	0,000	8608,907	8762,096

Therefore, we have:

$$a_1 = 26328{,}341,\ b_1 = 8610{,}653 \quad a_2 = -4356{,}913,\ b_2 = 8685{,}502$$

These estimates assume that the error in the values of *Concentration (%)* is constant, i.e., it is assumed that homoscedasticity exists. This means that all points $Area \times Concentration(\%)$ have equal weight in the calculation of estimates. Below, Cochran´s test will be applied to determine whether this assumption is valid.

Cochran´s C test

With the aim to evaluate the equality of variances of the dependent variable of the fitted model, Area, it was applied Cochran´s C test. This test was applied separately when:

- the analyte is the only component of the sample, having been obtained a p-value=0.005<0.05, leading to reject the null hypothesis of homogeneity of variances. The risk of rejecting the null hypothesis H_0 when it is true is less than 0.51%.

C (Observed value)	0,700
C (Critical value)	0,561
p-value (unilateral)	0,005
alfa	0,05

- The analyte is contained in a complex matrix, having been obtained a p-value=0.360>0.05, which does not reject the hypothesis of homogeneity of variances.

C (Observed value)	0,390
C (Critical value)	0,561
p-value (unilateral)	0,360
alfa	0,05

From this test it is concluded that only in the case that the analyte is the only component of the sample, the Intercept and Slope estimates of the regression line must be obtained again, but now using the Weighted Least Squares method.

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Adjusting the linear regression lines using the Weighted Least Squares method

Using the Cochran´s C test it was concluded that heteroscedasticity exists in the case where the analyte is the only component of the sample, which led to the need for, only in this case, using the weighted least squares method to re-estimate the Intercept and Slope of the regression line. By this method different weights are assigned to the observations, when determining the estimate of the regression line coefficients. Thus, the new estimates are as follows:

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	24570,318	4976,357	4,937	0,000	14154,683	34985,954
Conc. (%)	8589,312	38,558	222,763	0,000	8508,609	8670,016

The estimates, when the analyte is contained in a complex matrix, remain the same:

$$a_1 = 24570{,}318,\ b_1 = 8589{,}312 \quad a_2 = -4356{,}913,\ b_2 = 8685{,}502$$

ANOVA and the Coefficient of Determination (R^2)

Analysis of variance, ANOVA, aims to evaluate whether the adjusted regression line explains in a satisfactory way the relationship between Area and Concentration (%). In ANOVA, the F test is used to test whether the slopes of each estimated lines, b_1 and b_2, differs from zero. The coefficient of determination, R^2, gives the proportion of variability in the data that is properly explained by the regression line.

When the analyte is the only component of the sample, ANOVA was conducted considering weighted least squares method,

ANOVA

	SS	*df*	*MS*	*F*	*p-value*
Regression	817230938493	1	817230938493	49623,31	0,000
Residual	312905097	19	16468689		
Total	817543843590	20			

Regression Statistics	
R Square	0,9996
Adjusted R Square	0,9996
Standard Error	4058,16
Observations	21

This leads to the conclusion that the hypothesis of slope being zero is rejected p=0.000<0.05, and on the other hand, the line may explain 99.96% of the variability in the data.

When the analyte is contained in a complex matrix, ANOVA was conducted considering ordinary least squares method,

ANOVA

	SS	*df*	*MS*	*F*	*p-value*
Regression	1749602787503	1	1749602787503	56330,51	0,000
Residual	590132308	19	31059595		
Total	1750192919811	20			

Regression Statistics	
R Square	0,9997
Adjusted R Square	0,9996
Standard Error	5573,11
Observations	21

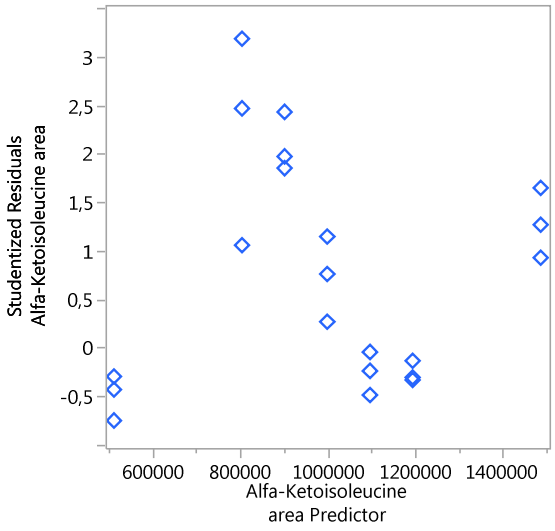
This leads to the conclusion that the hypothesis of slope being zero is rejected p=0.000<0.05, and on the other hand, the line may explain 99.97% of the variability in the data.

Residuals

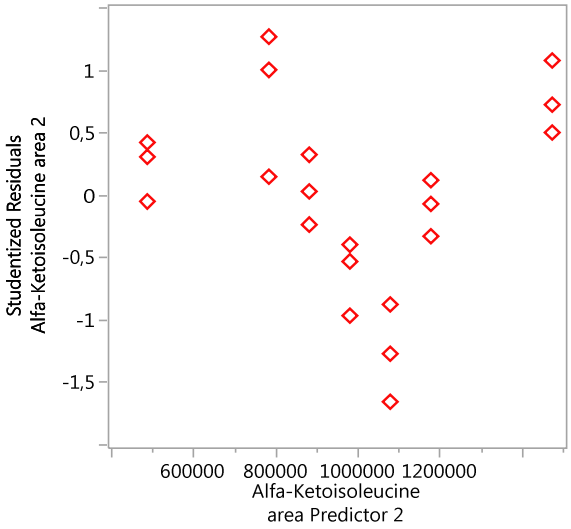
Residuals are extremely useful to check whether a particular linear regression model is appropriate for the data. So a scatterplot graph must be built in which residuals and predictive values for Area are crossed. For the regression line to be appropriate is necessary that the scatterplot does not produce a marked pattern.

The scatterplots for the two estimated regression lines are:

- the analyte is the only component of the sample,



- the analyte is contained in a complex matrix,



It is concluded that the lines are appropriate to the data and the estimates obtained from this are reliable. Thus there is good quality of fit of the regression lines.

Parallelism F test

The parallelism test aims to test the null hypothesis of equality of the slopes of the two estimated regression lines. It is intended to test the null hypothesis $b_1 = b_2$ *versus* the alternative hypothesis $b_1 \neq b_2$. If the null hypothesis is not rejected, it can be concluded that the effect of the matrix components is not significant.

The estimates obtained are:

$$b_1 = 8589{,}312 \quad \text{and} \quad b_2 = 8685{,}502$$

The result obtained by the test application is,

Paralell Fit SSE	*Full SSE*	*NDF*	*DDF*	*F Ratio*	*Prob > F*
971939017,50	902626184,81	1	38	2,918	0,096

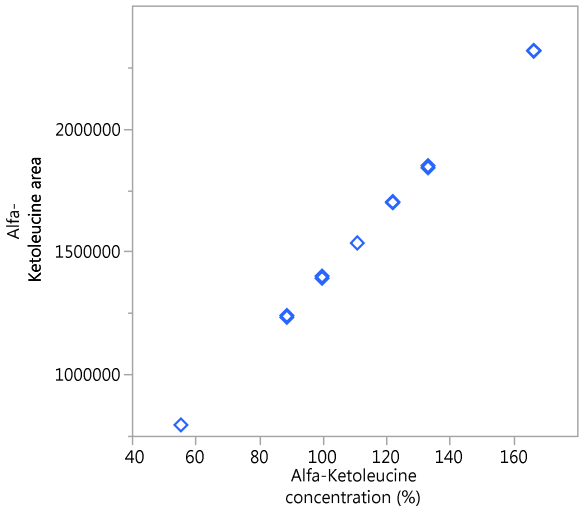
Thus, from the Parallelism F test, it was concluded that the equality of the slopes of the regression lines is not rejected, p=0.096>0.05; which allows to state that there is no statistical evidence (at the 5% level), to assert that the matrix has a significant effect.

Alfa-ketoleucine

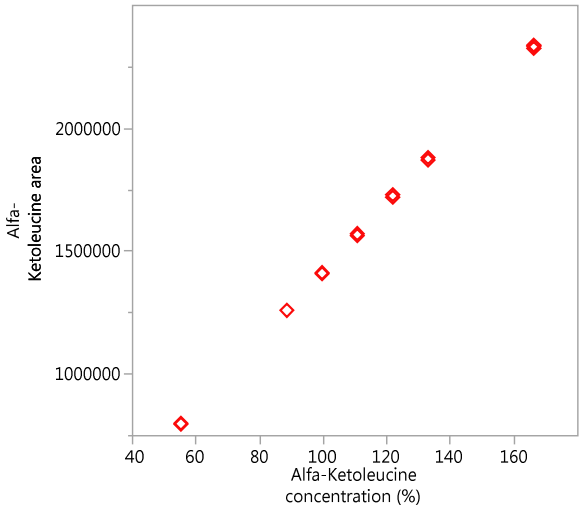
Scatterplots

In order to assess whether there is a linear relationship between the Concentration (%) and the Area of the analyte two scatterplots were constructed, one in w hich the analyte is the only

component of the sample, and another in which the analyte is in a complex matrix with other constituents.
Three different areas were observed for each of the seven concentrations.
In the case where the analyte is the only component of the sample, the following scatterplot shows a nearly perfect linear relationship.



It is also observed an almost perfect linear relationship between Area and Concentration (%) when the analyte is contained in a complex matrix.



Pearson´s correlation coefficient

Considering the correlation revealed in the two scatterplots, Pearson's correlation coefficients were estimated in order to understand if these were higher than the minimum acceptable, 0.990, which was verified. Thus, it was found that in both cases this assumption is satisfied because when the analyte is the only component of the sample r=0.9998 and when is in a complex matrix with other constituents r=0.9999.

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Adjusting the linear regression lines using the Least Squares method

After having been verified a linear correlation between the two variables, through a scatterplot and its Pearson's correlation coefficient (r≥0.990), two linear regression lines were estimated, one when the analyte is the only component of the sample,

$$Area = a_1 + b_1 \times Concentration(\%)$$

And another when the analyte is contained in a complex matrix,

$$Area = a_2 + b_2 \times Concentration(\%)$$

Intercepts, a_1 and a_2, and slopes, $b_1$and b_2, estimates were obtained from the method of least squares.

In the first case,

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	21499,873	6403,126	3,358	0,003	8097,976	34901,770
Conc. (%)	13738,482	55,399	247,990	0,000	13622,530	13854,434

In the second case,

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	28614,889	4001,802	7,151	0,000	20239,021	36990,757
Conc. (%)	13848,657	34,623	399,982	0,000	13776,190	13921,124

Therefore, we have:

$$a_1 = 21499{,}873,\ b_1 = 13738{,}482 \qquad a_2 = 28614{,}889,\ b_2 = 13848{,}657$$

These estimates assume that the error in the values of *Concentration (%)* is constant, i.e., it is assumed that homoscedasticity exists. This means that all points $Area \times Concentration(\%)$ have equal weight in the calculation of estimates. Below, Cochran´s test will be applied to determine whether this assumption is valid.

Cochran´s C test

Cochran´s C test was applied with the aim to evaluate the equality of variances of the dependent variable of the fitted model, Area.

In both situations, whether the analyte is the only component or contained in a matrix, the homoscedasticity hypothesis was not rejected, respectively, p=0.317 and p=0.910.

Therefore, it is concluded that the estimates found by the method of ordinary least squares are valid. The following tables show test results:

- in the first case,

C (Observed value)	0,403
C (Critical value)	0,561
p-value (unilateral)	0,317
alfa	0,05

- in the second case,

C (Observed value)	0,288
C (Critical value)	0,561
p-value (unilateral)	0,910
alfa	0,05

ANOVA and the Coefficient of Determination (R^2)

Analysis of variance, ANOVA, aims to evaluate whether the adjusted regression line explains in a satisfactory way the relationship between Area and Concentration (%). In ANOVA, the F test is used to test whether the slopes of each estimated lines, b_1 and b_2, differs from zero. The coefficient of determination, R^2, gives the proportion of variability in the data that is properly explained by the regression line.
For the analyte to be the only component we have:

ANOVA

	SS	*df*	*MS*	*F*	*p-value*
Regression	4180330786400	1	4180330786400	61499,22	0,000
Residual	1291500708	19	67973721		
Total	4181622287108	20			

Regression Statistics	
R Square	0,9997
Adjusted R Square	0,9997
Standard Error	8244,62
Observations	21

This leads to the conclusion that the hypothesis of slope being zero is rejected p=0.000<0.05, and on the other hand, the line may explain 99.97% of the variability in the data.

In case the analyte is contained in a complex matrix, ANOVA was conducted considering ordinary least squares method,

ANOVA

	SS	*df*	*MS*	*F*	*p-value*
Regression	4247647690869	1	4247647690869	159985,4	0,000
Residual	504454237	19	26550223		
Total	4248152145107	20			

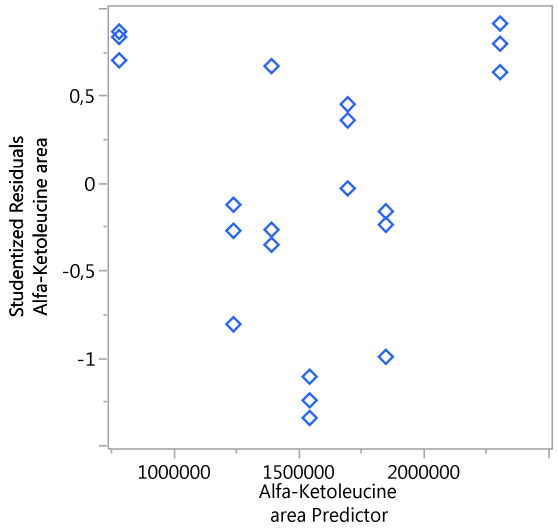
Regression Statistics	
R Square	0,9999
Adjusted R Square	0,9999
Standard Error	5152,69
Observations	21

This leads to the conclusion that the hypothesis of slope being zero is rejected p=0.000<0.05, and on the other hand, the line may explain 99.99% of the variability in the data.

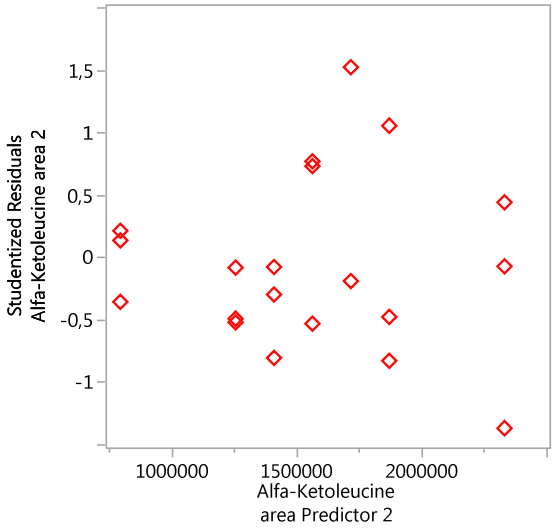
Residuals

Residuals are extremely useful to check whether a particular linear regression model is appropriate for the data. So a scatterplot must be built in which residuals and predictive values for Area are crossed. For the regression line to be appropriate is necessary that the scatterplot does not produce a marked pattern.
The scatterplots for the two estimated regression lines are:

- the analyte is the only component of the sample,



- the analyte is contained in a complex matrix ,



The scatterplots show that there is good quality fit of regression lines.

Parallelism F test

The parallelism test aims to test the null hypothesis of equality of the slopes of the two estimated regression lines. It is intended to test the null hypothesis $b_1 = b_2$ *versus* the alternative hypothesis $b_1 \neq b_2$. If the null hypothesis is not rejected, it can be concluded that the effect of the matrix components is not significant.
The estimates obtained are:

$$b_1 = 13738{,}482 \text{ and } \quad b_2 = 13848{,}657$$

The following table indicates the test results,

Paralell Fit SSE	*Full SSE*	*NDF*	*DDF*	*F Ratio*	*Prob > F*
1929450165,60	1795031104,60	1	38	2,846	0,100

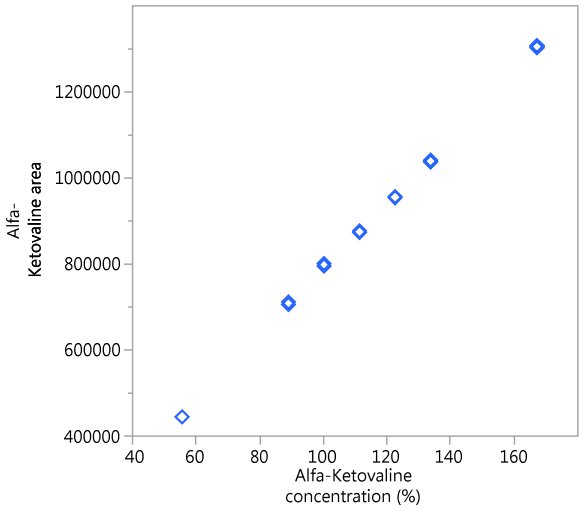
These results show that the equality of the slopes of the regression lines is not rejected, p=0.100>0.05; therefore, no statistical evidence exists at the 5% level to assert that the matrix has a significant effect.

Alfa-ketovaline

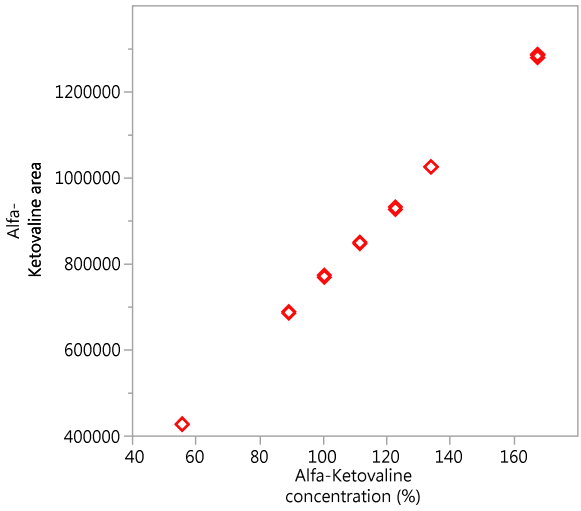
Scatterplots

Also for this analyte was evaluated the existence of a linear relationship between the Concentration (%) and Area through the construction of two scatterplots, wherein one addresses the case where the analyte is the only component of the sample, and the other when it is contained in a complex matrix.
Similarly, three different areas were observed for each of the seven concentrations.

In the first case the following scatter chart reveals an almost perfect linear relationship,



The same goes for the second case,



Pearson´s correlation coefficient

Taking into account the correlation revealed in the two previous figures, we determined the Pearson´s correlation coefficients for both cases. When the analyte is the only component of the sample, r=0.9998 and when it´s contained in a matrix, r=0.9998. The two estimated coefficients are above the minimum acceptable value, 0.990.

Adjusting the linear regression lines using the Least Squares method

After having been verified a linear correlation between the two variables, through a scatter plot and its Pearson's correlation coefficient ($r \geq 0.990$), two linear regression lines were estimated, one when the analyte is the only component of the sample,

$$Area = a_1 + b_1 \times Concentration(\%)$$

And another when the analyte is contained in a complex matrix,

$$Area = a_2 + b_2 \times Concentration(\%)$$

Intercepts, a_1 and a_2, and slopes, b_1 and b_2, estimates were obtained from the method of least squares.
When the analyte is the only component of the sample, the following results from the regression straight line fit were obtained,

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	18889,556	4504,537	4,193	0,000	9461,451	28317,660
Conc. (%)	7656,629	38,736	197,662	0,000	7575,554	7737,705

When the analyte is contained in a complex matrix,

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-1753,722	4081,396	-0,430	0,672	-10296,181	6788,737
Conc. (%)	7638,167	35,057	217,877	0,000	7564,792	7711,543

The estimates are:

$$a_1 = 18889{,}556,\ b_1 = 7656{,}629 \quad a_2 = -1753{,}722,\ b_2 = 7638{,}167$$

The estimates obtained above assume that the error in the values of Concentration (%) is constant, therefore homoscedasticity exists. Thus, in order to take into account these estimates, Cochran´s C test must be applied to assess whether the homogeneity assumption is not violated.

Cochran´s C test

Like the previous analyte, here also the equality of variances of the dependent variable of the fitted model was evaluated and the Cochran´s C test was applied as indicated in the table below. In both situations, when analytics is isolated in the sample, and when it is inserted in a matrix, the hypothesis of homoscedasticity was not rejected, respectively p=0.713 and p=0.732.
Therefore, it is concluded that the estimates found by the method of ordinary least squares are valid. The following tables present the results of the tests:

- the analyte is the only component of the sample,

C (Observed value)	0,317
C (Critical value)	0,561
p-value (unilateral)	0,713
alfa	0,05

- the analyte is contained in a complex matrix,

C (Observed value)	0,314
C (Critical value)	0,561
p-value (unilateral)	0,732
alfa	0,05

ANOVA and the Coefficient of Determination (R^2)

Analysis of variance, ANOVA, aims to assess whether the fitted regression line satisfactorily explains the relationship between Area and Concentration (%). In this analysis of variance, the F test is applied in order to test whether the slope of each of the estimated lines, b_1 and b_2, differs from zero. Meanwhile, the coefficient of determination, R^2, gives the proportion of the variability of the observed data that is properly explained by the estimated regression line. In case of analyte being the only component of the sample we have,

ANOVA

	SS	*df*	*MS*	*F*	*p-value*
Regression	1314323198253	1	1314323198253	39070,13	0,000
Residual	639161885	19	33640099		
Total	1314962360138	20			

Regression Statistics	
R Square	0,9995
Adjusted R Square	0,9995
Standard Error	5800,01
Observations	21

This leads to the conclusion that the hypothesis of slope being zero is rejected p=0.000<0.05, and on the other hand, the line may explain 99.95% of the variability in the data.
When the analyte is contained in a complex matrix,

ANOVA

	SS	*df*	*MS*	*F*	*p-value*
Regression	1310977207284	1	1310977207284	47470,17	0,000
Residual	524720375	19	27616862		
Total	1311501927659	20			

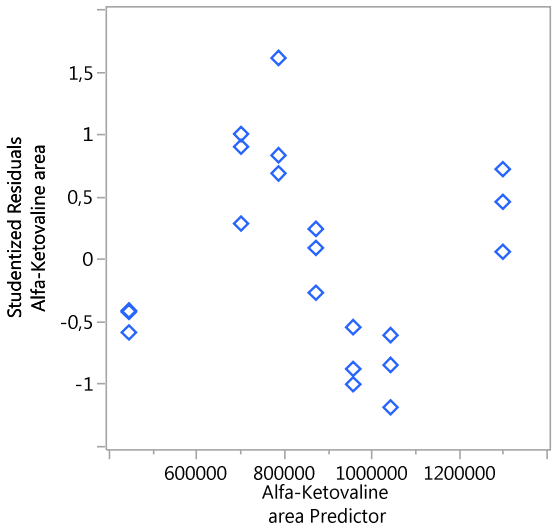
Regression Statistics	
R Square	0,9996
Adjusted R Square	0,9996
Standard Error	5255,17
Observations	21

Therefore, the hypothesis of slope being zero is rejected p=0.000<0.05, and the line represents 99.96% of the variability in the data.

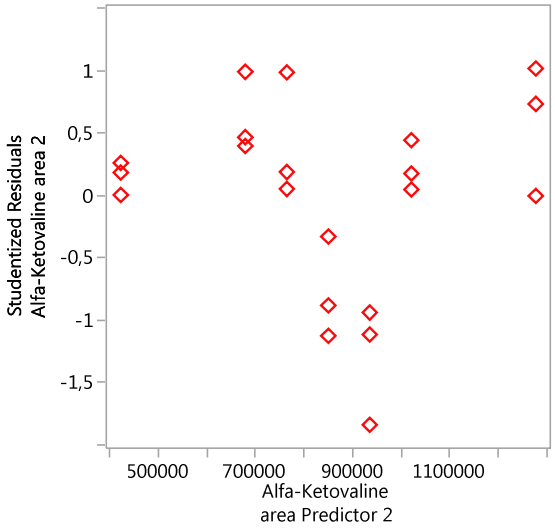
Residuals

Residuals are extremely useful to check whether a particular linear regression model is appropriate for the data. So a scatterplot graph must be built in which residuals and predictive values for Area are crossed. For the regression line to be appropriate is necessary that the scatterplot does not produce a marked pattern.
The scatterplots for the two estimated regression lines are:

- the analyte is the only component of the sample,



- the analyte is contained in a complex matrix,



Like the previous analyte, the two scatterplots show that there is good quality fit of regression lines.

Parallelism F test

The parallelism test aims to test the null hypothesis of equality of the slopes of the two estimated regression lines. It is intended to test the null hypothesis $b_1 = b_2$ *versus* the alternative hypothesis $b_1 \neq b_2$. If the null hypothesis is not rejected, it can be concluded that the effect of the matrix components is not significant.

The estimates obtained are:

$$b_1 = 7656{,}629 \qquad \text{and} \qquad b_2 = 7638{,}167$$

The following table indicates the test results,

Paralell Fit SSE	*Full SSE*	*NDF*	*DDF*	*F Ratio*	*Prob > F*
1168038041,50	1164186912,80	1	38	0,126	0,725

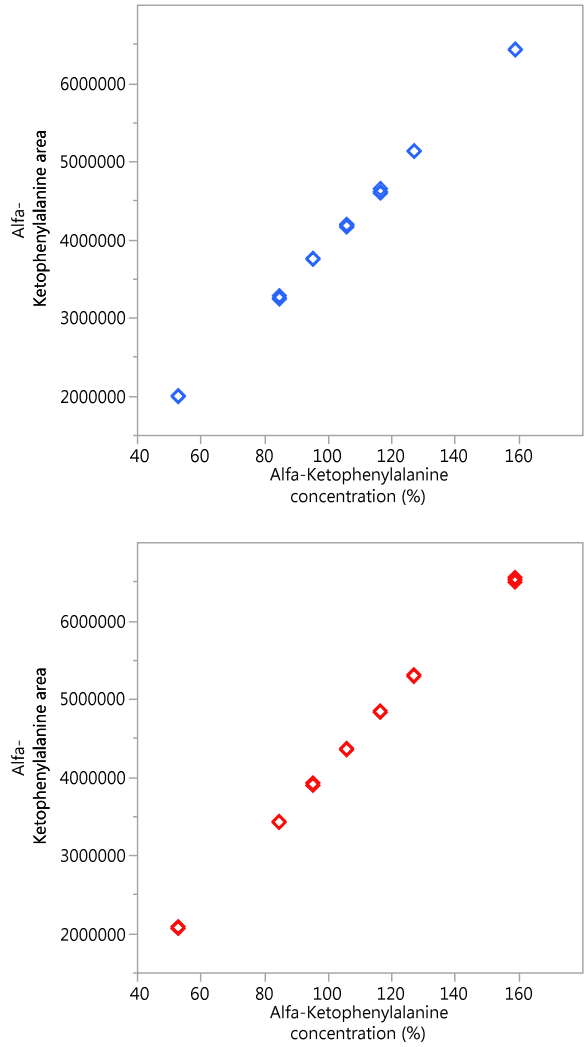
These results show that the equality of the slopes of the regression lines is not rejected, p=0.725>0.05; therefore, no statistical evidence exists at the 5% level to assert that the matrix has a significant effect.

Alfa-ketophenylalanine

Scatterplots

The first step is to evaluate the existence of a linear relationship between the Concentration (%) and the Area of the analyte. For this, two scatterplots were constructed, one for each case.

Three different areas were observed for each of the seven concentrations.



Above we can see that in both cases the scatterplots reveal an almost perfect linear relationship.

Pearson´s correlation coefficient

Taking into account the success of the above visual relation between variables, it was determined the Pearson´s correlation coefficients in order to understand if this ratio is higher than the minimum acceptable, 0.990, which was found:
Analyte is the only component of the sample: r=0.9997
Analyte is contained in a matrix: r=0.9995

Adjusting the linear regression lines using the Least Squares method

After having been verified a linear correlation between the two variables, through a scatter plot and its Pearson's correlation coefficient (r≥0.990), two linear regression lines were estimated, one when the analyte is the only component of the sample,

$$Area = a_1 + b_1 \times Concentration(\%)$$

And another when the analyte is contained in a complex matrix,

$$Area = a_2 + b_2 \times Concentration(\%)$$

Intercepts, a_1 and a_2, and slopes, b_1 and b_2, estimates were obtained from the method of least squares.
In the following two tables we have the results,

- the analyte is the only component of the sample,

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-276630,20	25297,169	-10,935	0,000	-329577,79	-223682,62
Conc. (%)	42155,491	229,046	184,048	0,000	41676,093	42634,890

- the analyte is contained in a complex matrix,

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-140147,00	32643,363	-4,293	0,000	-208470,35	-71823,66
Conc. (%)	42315,917	295,714	143,097	0,000	41696,981	42934,854

Where the following estimates were extracted:

$$a_1 = -276630{,}20,\ b_1 = 42155{,}491 \qquad a_2 = -140147{,}00,\ b_2 = 42315{,}917$$

The estimates obtained above assume that the error in the values of Concentration (%) is constant, therefore homoscedasticity exists. Which means that all the points $Area \times Concentration(\%)$ have equal weight in the calculation of estimates. However, Cochran´s C test must be applied to assess whether the homogeneity assumption is not violated.

Cochran´s C test

As in previous analytes this test was applied when:

- the analyte is the only component of the sample,

C (Observed value)	0,429
C (Critical value)	0,561
p-value (unilateral)	0,242
alfa	0,05

- the analyte is contained in a complex matrix,

C (Observed value)	0,466
C (Critical value)	0,561
p-value (unilateral)	0,162
alfa	0,05

As Cochran´s C test does not reject the hypothesis of homogeneity of variances, p=0.242 and p=0.162, it is concluded that the estimates found by the method of ordinary least squares are valid for both cases.

ANOVA and the Coefficient of Determination (R^2)

This analysis aims to assess whether the fitted regression line satisfactorily explains the relationship between Area and Concentration (%). In this analysis of variance, the F test is applied in order to test whether the slope of each of the estimated lines, b_1 and b_2, differs from zero. Meanwhile, the coefficient of determination, R^2, gives the proportion of the variability of the observed data is properly explained by the estimated regression line.
ANOVA results for the two cases are given by the following tables:

ANOVA

	SS	*df*	*MS*	*F*	*p-value*
Regression	35938844321344	1	35938844321344	33873,75	0,000
Residual	20158323689	19	1060964405		
Total	35959002645033	20			

ANOVA

	SS	**df**	**MS**	**F**	**p-value**
Regression	36175169513017	1	36175169513017	20476,88	0,000
Residual	33566057748	19	1766634618		
Total	36208735570765	20			

The estimates differ significantly from zero, p=0.000<0.05, both in the case of the analyte being the only component of the sample either when it is contained in a complex matrix.
It is also important to point that the coefficient of determination, R^2, which gives us the proportion of variability of the observed data, is properly explained by the estimated regression line.

The R^2 value for the case where the analyte is isolated is 0.9994.

Regression Statistics	
R Square	0,9994
Adjusted R Square	0,9994
Standard Error	32572,45
Observations	21

In the case where it is contained in a matrix is 0.9991.

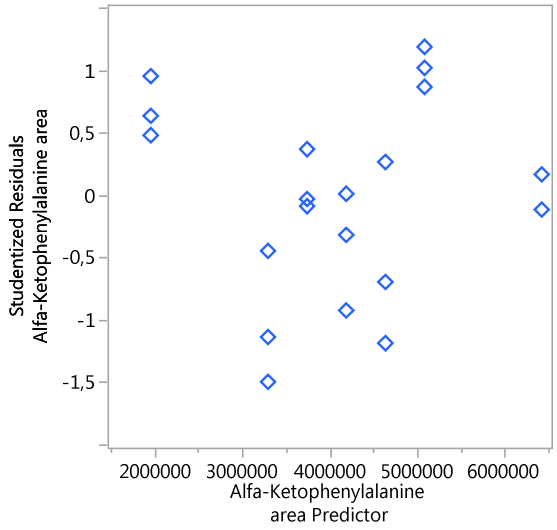
Regression Statistics	
R Square	0,9991
Adjusted R Square	0,9990
Standard Error	42031,35
Observations	21

This means that, in the first case, 99.94% of the variability of the data is explained by the line; while in the second case the percentage is 99.91%, which is an exceptional value.

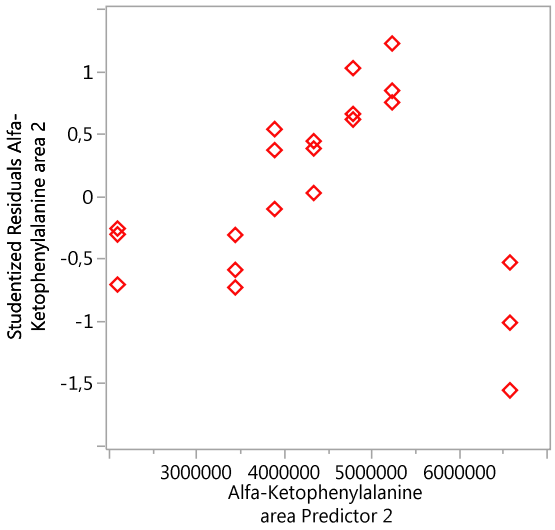
Residuals

Residuals allow you to check the quality of the fit of a linear regression model. So a scatter graph must be built in which residuals and predictive values for Area are crossed. The regression line is well adjusted when the graph does not produce a marked pattern.
The scatterplots for the two estimated regression lines are:

- the analyte is the only component of the sample,



- the analyte is contained in a complex matrix,



From the above graphs it is concluded that there is a good quality fit of the regression lines.

Parallelism F test

The parallelism test aims to test the null hypothesis of equality of the slopes of the two estimated regression lines. It is intended to test the null hypothesis $b_1 = b_2$ *versus* the alternative hypothesis $b_1 \neq b_2$. If the null hypothesis is not rejected, it can be concluded that the effect of the matrix components is not significant.
Estimates previously obtained are:

$$b_1 = 42155{,}491 \text{ and } \quad b_2 = 42315{,}917$$

The result of the Parallelism F Test is given in the following table:

Paralell Fit SSE	Full SSE	NDF	DDF	F Ratio	Prob > F
53987140666	53726270477	1	38	0,185	0,670

At the 5% significance level, it can be concluded by the Parallelism F test, that the equality of the regression lines slopes is not rejected, p=0.670>0.05; which means that there is no statistical evidence to say that the matrix has a significant effect.

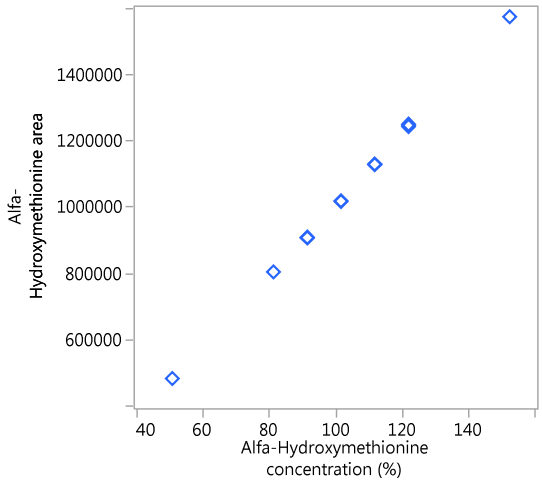
Alfa-Hydroxymethionine

Scatterplots

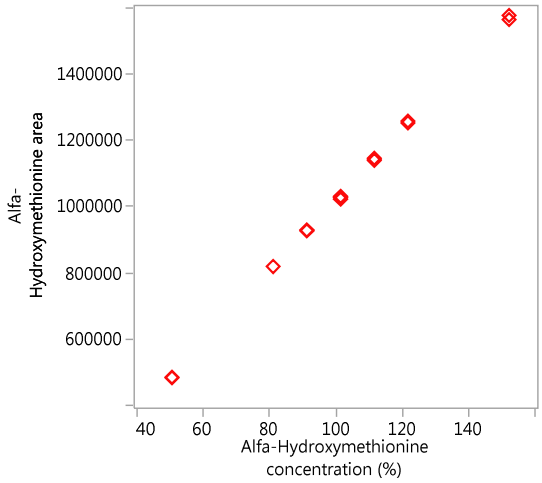
Similar to what was done for the previous analytes, the existence of a linear relationship between the Concentration (%) and Area was evaluated through the construction of two scatterplots,

wherein one addresses the case where the analyte is the only component of the sample, and the other when it is contained in a complex matrix. Again, three different areas were observed for each of the seven concentrations.

1st case scenario: the scatterplot reveals an almost perfect linear relationship,



2nd scenario: there is also an almost perfect linear relationship,



Pearson´s correlation coefficient

Taking into account the correlation revealed in the two previous figures, we determined the Pearson´s correlation coefficients for both cases. When the analyte is the only component of the sample, r=0.9999 and when it´s contained in a matrix, r=0.9999. The two estimated coefficients are above the minimum acceptable value, 0.990.

Adjusting the linear regression lines using the Least Squares method

As the linear correlation between the two variables was observed through a scatterplot and it´s Pearson's correlation coefficient (r≥0.990), two linear regression lines were estimated, one when the analyte is the only component of the sample,

$$Area = a_1 + b_1 \times Concentration(\%)$$

And another when the analyte is contained in a complex matrix,

$$Area = a_2 + b_2 \times Concentration(\%)$$

Intercepts, a_1 and a_2, and slopes, b_1 and b_2, estimates were obtained from the method of least squares.
The following results from the regression straight line fit were obtained:

- analyte is the only component of the sample:

	Coefficients	Std. Error	t Stat	P-value	Lower 95%	Upper 95%
Intercept	-74241,746	4194,556	-17,700	0,000	-83021,053	-65462,439
Conc. (%)	10770,977	39,561	272,261	0,000	10688,174	10853,780

- analyte is contained in a complex matrix:

	Coefficients	Std. Error	t Stat	P-value	Lower 95%	Upper 95%
Intercept	-58860,151	4107,124	-14,331	0,000	-67456,460	-50263,842
Conc. (%)	10722,086	38,788	276,429	0,000	10640,902	10803,270

In short,

$$a_1 = -74241{,}746,\ b_1 = 10770{,}977 \qquad a_2 = -58860{,}151,\ b_2 = 10722{,}086$$

These estimates assume that the error in the values of Concentration (%) is constant, therefore homoscedasticity exists. Which means that all the points $Area \times Concentration(\%)$ have equal weight in the calculation of estimates.

Cochran´s C test

Cochran´s C test must be applied to assess whether the homogeneity assumption is not violated. As in previous scenarios, this test was applied taking into account the two situations already described, and confirmed that the null hypothesis of homoscedasticity is not rejected, p=0.073 and p=0.099, in accordance with the following results:

- the analyte is the only component of the sample,

C (Observed value)	0,533
C (Critical value)	0,561
p-value (unilateral)	0,073
alfa	0,05

- the analyte is contained in a matrix,

C (Observed value)	0,508
C (Critical value)	0,561
p-value (unilateral)	0,099
alfa	0,05

From this conclusion the estimates obtained by the least squares method to the parameters of both linear regression lines are accepted.

ANOVA and the Coefficient of Determination (R^2)

This analysis aims to assess whether the fitted regression line satisfactorily explains the relationship between Area and Concentration(%). In this analysis of variance, the F test is applied in order to test whether the slope of each of the estimated lines, b_1 and b_2, differs from zero.
The estimates differ significantly from zero, p = 0.000 <0.05, both when the analyte is a single component, as when inserted in a matrix. It is indicated in the following ANOVA tables:

ANOVA

	SS	df	MS	F	p-value
Regression	2162219926512	1	2162219926512	74126,06	0,000
Residual	554220445	19	29169497		
Total	2162774146957	20			

ANOVA

	SS	df	MS	F	p-value
Regression	2136974295515	1	2136974295515	76412,91327	0,000
Residual	531356676	19	27966141		
Total	2137505652191	20			

Meanwhile, the coefficient of determination, R^2, gives the proportion of the variability of the observed data is properly explained by the estimated regression line.

R^2 value is 0.9997 for the case where the analyte is isolated, meaning that 99.97% of data variability is explained by the line.
R^2 value is 0.9998 considering the other case, meaning that 99.98% of data variability is explained by the line:

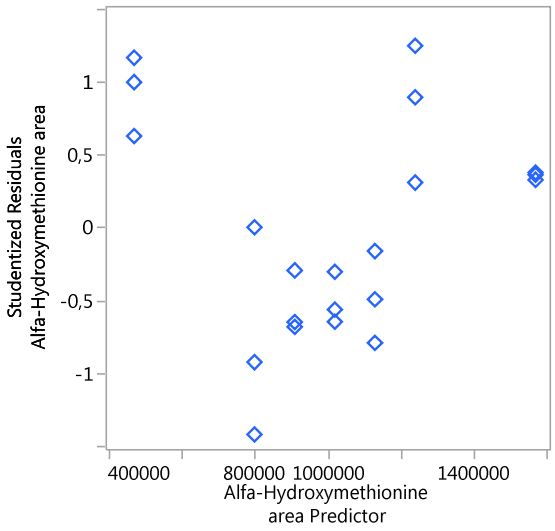
Regression Statistics	
R Square	0,9997
Adjusted R Square	0,9997
Standard Error	5400,88
Observations	21

Regression Statistics	
R Square	0,9998
Adjusted R Square	0,9997
Standard Error	5288,30
Observations	21

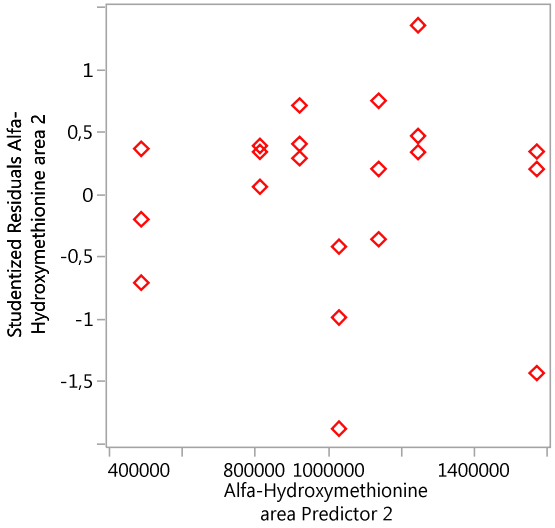
Residuals

Residuals allow you to check the quality of the fit of a linear regression model. So a scatter graph must be built in which residuals and predictive values for Area are crossed. The regression line is well adjusted when the graph does not produce a marked pattern.
The scatterplots for the two estimated regression lines are:

- the analyte is the only component of the sample,



- the analyte is contained in a complex matrix,



The above graphs show the good quality of the adjustment of the regression lines.

Parallelism F test

As already mentioned, the purpose of the parallel test is to test the null hypothesis of equality of the slopes of the two estimated regression lines. It is intended to test the null hypothesis $b_1 = b_2$ *versus* the alternative hypothesis $b_1 \neq b_2$. If the null hypothesis is not rejected, it can be concluded that the effect of the matrix components is not significant.
Estimates that had been reached:

$$b_1 = 10770{,}977 \text{ and } \quad b_2 = 10722{,}086$$

The results of the Parallelism F Test are:

Paralell Fit SSE	Full SSE	NDF	DDF	F Ratio	Prob > F
1108113557,60	1085834500,50	1	38	0,780	0,383

At the 5% significance level, it can be concluded by the Parallelism F test, that the equality of the regression lines slopes is not rejected, p=0.383; which means that there is no statistical evidence to say that the matrix has a significant effect.

3.3.4. Precision

3.3.4.1. Intermediate Precision

Acceptance criteria:

- RSD for the determinations of one analyst should not be higher than 3.7%
- RSD between the results of the two analysts should not be higher than 3.7%
- The average of two analysts should not be statistically different (*t Test*)

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

- Alfa-Ketoisoleucine

Sample	Analyst 1: SMP Date: 20 June 2016 Assay (%)	Analyst 2: MC Date: 22 June 2016 Assay (%)
A1	100.8	102.6
A2	100.8	99.7
A3	100.5	99.0
A4	99.7	101.4
A5	99.8	100.2
A6	100.4	99.6
Average (%)	**100.3**	**100.4**
RSD (%)	**0.5**	**1.3**

Mean between analysts: 100.4 %
RSD between analysts: 1.0 %

t Test
|t Stat| = -0.141888
t Critical two-tail = 2.570582
t Stat≤t Critical two-tail, the average between the two analysts are not statistically different.

- Alfa-Ketoleucine

Sample	Analyst 1: SMP Date: 20 June 2016 Assay (%)	Analyst 2: MC Date: 22 June 2016 Assay (%)
A1	101.5	101.8
A2	102.5	102.4
A3	102.4	102.3
A4	102.0	102.0
A5	101.8	102.0
A6	102.8	101.9
Average (%)	**102.2**	**102.1**
RSD (%)	**0.5**	**0.2**

Mean between analysts: 102.1%
RSD between analysts: 0.4 %

t Test
t Stat = 0.577350
t Critical two-tail = 2.570582
t Stat≤t Critical two-tail, the average between the two analysts are not statistically different.

- Alfa-Ketovaline

Sample	Analyst 1: SMP Date: 20 June 2016 Assay (%)	Analyst 2: MC Date: 22 June 2016 Assay (%)
A1	99.5	102.3
A2	99.7	99.4
A3	99.1	99.5
A4	98.4	100.9
A5	98.5	100.2
A6	98.9	99.5
Average (%)	**99.0**	**100.3**
RSD (%)	**0.5**	**1.1**

Mean between analysts: 99.7%
RSD between analysts: 1.1 %

t Test
|t Stat| = -2.531744
t Critical two-tail = 2.570582
t Stat≤t Critical two-tail, the average between the two analysts are not statistically different.

- Alfa-Ketophenylalanine

Sample	Analyst 1: SMP Date: 20 June 2016 Assay (%)	Analyst 2: MC Date: 22 June 2016 Assay (%)
A1	101.4	98.8
A2	102.3	103.4
A3	101.9	101.1
A4	102.1	100.3
A5	102.1	100.9
A6	103.3	101.9
Average (%)	**102.2**	**101.0**
RSD (%)	**0.6**	**1.5**

Mean between analysts: 101.6%
RSD between analysts: 1.2 %

t Test
t Stat = 2.194423
t Critical two-tail = 2.570582
t Stat≤t Critical two-tail, the average between the two analysts are not statistically different.

- Alfa-Hydroxymethionine

Sample	Analyst 1: SMP Date: 20 June 2016 Assay (%)	Analyst 2: MC Date: 22 June 2016 Assay (%)
A1	101.4	98.8
A2	101.9	102.7
A3	101.9	101.3
A4	101.6	100.5
A5	101.9	101.0
A6	102.3	101.1
Average (%)	**101.8**	**100.9**
RSD (%)	**0.3**	**1.3**

Mean between analysts: 101.4%
RSD between analysts: 1.0 %

t Test
t Stat = 2.088157
t Critical two-tail = 2.570582
t Stat≤t Critical two-tail, the average between the two analysts are not statistically different.

3.3.4.2. Repeatability

Acceptance criteria: RSD for 6 measurements should not be higher than 3.7%

- Alfa-Ketoisoleucine

Sample	Assay (%)
A1	100.8
A2	100.8
A3	100.5
A4	99.7
A5	99.8

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Sample	Assay (%)
A6	100.4
Average (%)	**100.3**
RSD (%)	**0.5**

- Alfa-Ketoleucine

Sample	Assay (%)
A1	101.5
A2	102.5
A3	102.4
A4	102.0
A5	101.8
A6	102.8
Average (%)	**102.2**
RSD (%)	**0.5**

- Alfa-Ketovaline

Sample	Assay (%)
A1	99.5
A2	99.7
A3	99.1
A4	98.4
A5	98.5
A6	98.9
Average (%)	**99.0**
RSD (%)	**0.5**

- Alfa-Ketophenylalanine

Sample	Assay (%)
A1	101.4
A2	102.3
A3	101.9
A4	102.1
A5	102.1

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Sample	Assay (%)
A6	103.3
Average (%)	**102.2**
RSD (%)	**0.6**

- Alfa-Hydroxymethionine

Sample	Assay (%)
A1	101.4
A2	101.9
A3	101.9
A4	101.6
A5	101.9
A6	102.3
Average (%)	**101.8**
RSD (%)	**0.3**

3.3.4.3. Instrumental Repeatability

Acceptance criteria: RSD between the areas of 10 injections should not be higher than 3.7%

Injection	Alfa-Ketoisoleucine Area	Alfa-Ketoleucine Area	Alfa-Ketovaline Area
1	920611	1246778	801057
2	916065	1243664	802438
3	918256	1245349	799059
4	911661	1242787	800174
5	915412	1240923	799771
6	915778	1238366	796902
7	910728	1244465	795308
8	916360	1247626	797267
9	913075	1244754	796291
10	910032	1247196	794273
Average	**914798**	**1244191**	**798254**
RSD (%)	**0.4**	**0.2**	**0.3**

Injection	Alfa-Ketophenylalanine Area	Alfa-Hydroxymethionine Area
1	3830985	1024696
2	3840727	1024525
3	3842200	1024779
4	3875800	1022751
5	3870039	1025497
6	3878309	1021805
7	3890728	1019418
8	3894505	1032149
9	3899089	1030446
10	3914252	1027431
Average	**3873664**	**1025350**
RSD (%)	**0.7**	**0.4**

3.3.5. Accuracy

Acceptance criteria:

- Recovery percentage should be between 97% and 103%
- RSD between the results for three determinations of each level must be equal or less than 3.7%

- Alfa-Ketoisoleucine

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
80	81.309	79.935	98.311
	81.309	80.081	98.490
	82.379	80.032	97.152
	Mean (%)		97.984
	RSD (%)		0.741
100	101.636	98.969	97.376
	102.171	99.329	97.218
	102.171	99.396	97.284
	Mean (%)		97.293
	RSD (%)		0.082
120	121.963	121.003	99.213
	122.498	121.869	99.486
	122.498	121.161	98.908

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
	Mean (%)		99.202
	RSD (%)		0.291

- Alfa-Ketoleucine

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
80	79.309	78.342	98.782
	79.664	79.995	100.415
	80.020	79.803	99.729
	Mean (%)		99.642
	RSD (%)		0.823
100	99.225	99.547	100.325
	99.580	100.494	100.918
	99.580	98.852	99.268
	Mean (%)		100.170
	RSD (%)		0.834
120	119.852	119.615	99.802
	119.852	119.361	99.591
	119.496	118.763	99.387
	Mean (%)		99.593
	RSD (%)		0.209

- Alfa-Ketovaline

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
80	81.868	81.225	99.215
	81.444	81.202	99.703
	81.444	81.173	99.668
	Mean (%)		99.528
	RSD (%)		0.274
100	102.229	100.978	98.776
	101.805	101.588	99.787
	102.229	101.367	99.157
	Mean (%)		99.240

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
	RSD (%)		0.514
120	122.590	122.601	100.009
	122.166	122.238	100.059
	122.166	122.106	99.952
	Mean (%)		100.007
	RSD (%)		0.054

- Alfa-Ketophenylalanine

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
80	80.091	77.968	97.349
	79.539	78.886	99.179
	78.986	77.572	98.209
	Mean (%)		98.246
	RSD (%)		0.932
100	99.424	99.256	99.831
	99.976	99.364	99.388
	99.424	98.302	98.872
	Mean (%)		99.364
	RSD (%)		0.483
120	119.308	116.322	97.497
	118.756	116.107	97.769
	118.756	115.473	97.235
	Mean (%)		97.501
	RSD (%)		0.274

- Alfa-Hydroxymethionine

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
80	80.705	79.483	98.485
	80.033	78.176	97.680
	80.705	78.750	97.577
	Mean (%)		97.914
	RSD (%)		0.508

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
100	100.881	98.440	97.580
	100.881	99.006	98.141
	101.554	99.777	98.250
	Mean (%)		97.990
	RSD (%)		0.367
120	121.058	120.362	99.425
	121.058	118.616	97.983
	122.403	121.426	99.202
	Mean (%)		98.870
	RSD (%)		0.785

3.3.6. Robustness

3.3.6.1. Sample and standard solutions stability

Acceptance criteria: The sample and standard solutions are considered stable if the RSD between the areas of injections of the sample and the standard is less than 2,5% in the period tested.

→ **Sample solution**

- Alfa-Ketoisoleucine

Time point	Area
0 hour	921159
24 hours	917932
48 hours	919618
Average (0–48 hours)	**920389**
RSD (0–48 hours)	**0.1 %**

- Alfa-Ketoleucine

Time point	Area
0 hour	1338724
24 hours	1322010
48 hours	1314621
Average (0–48 hours)	**1326673**
RSD (0–48 hours)	**1.3 %**

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

- Alfa-Ketovaline

Time point	Area
0 hour	794921
24 hours	792962
48 hours	793388
Average (0–48 hours)	**794155**
RSD (0–48 hours)	**0.1 %**

- Alfa-Ketophenylalanine

Time point	Area
0 hour	3664608
24 hours	3735276
48 hours	3734956
Average (0–48 hours)	**3699782**
RSD (0–48 hours)	**1.3 %**

- Alfa-Hydroxymethionine

Time point	Area
0 hour	1056621
24 hours	1067130
48 hours	1068656
Average (0–48 hours)	**1062639**
RSD (0–48 hours)	**0.8 %**

The sample solution is stable during 48 hours after preparation.

→ **Standard solution**

- Alfa-Ketoisoleucine

Time point	Area
0 hour	914274
24 hours	919764
48 hours	917657
Average (0–48 hours)	**915966**

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Time point	Area
RSD (0–48 hours)	**0.3 %**

- Alfa-Ketoleucine

Time point	Area
0 hour	1296259
24 hours	1282257
48 hours	1276364
Average (0–48 hours)	**1284960**
RSD (0–48 hours)	**0.8 %**

- Alfa-Ketovaline

Time point	Area
0 hour	792624
24 hours	794295
48 hours	794998
Average (0–48 hours)	**793811**
RSD (0–48 hours)	**0.2 %**

- Alfa-Ketophenylalanine

Time point	Area
0 hour	3643037
24 hours	3713741
48 hours	3716470
Average (0–48 hours)	**3679754**
RSD (0–48 hours)	**1.4 %**

- Alfa-Hydroxymethionine

Time point	Area
0 hour	1051077
24 hours	1057673
48 hours	1065652
Average (0–48 hours)	**1058365**
RSD (0–48 hours)	**1.0 %**

The standard solution is stable during 48 hours after preparation.

3.3.6.2. Variation of the mobile phase composition

The mobile phase composition was changed in the range of 0.024M to 0.026M Sulfuric acid (0.025M ± 0.001M), determining their influence on the retention times and resolution when compared with the original method conditions.

Variation of mobile phase composition	0.025 M sulfuric acid (method conditions)		0.024 M sulfuric acid		0.026 M sulfuric acid	
	rt (min)	Resol.	rt (min)	Resol.	rt (min)	Resol.
Alfa-Ketoisoleucine	20.810	3.233	20.721	2.163	20.931	2.360
Alfa-Ketoleucine	24.323	1.649	24.206	1.281	24.555	1.311
Alfa-Ketovaline	15.260	6.504	15.256	4.342	15.274	4.619
Alfa-Ketophenylalanine	31.230	3.362	31.206	2.272	31.451	2.347
Alfa-Hydroxymethionine	26.186	3.362	26.328	2.272	26.599	2.347
Comment	-------		Not critical: slight variation of retention times and decrease of resolution (in particular for ketovaline), nevertheless the acceptance criteria (not less than 1) is fulfilled.		Not critical: slight variation of retention times and decrease of resolution (in particular for ketovaline, nevertheless the acceptance criteria (not less than 1) is fulfilled.	

3.3.6.3. Variation of the flow rate

The flow rate was changed in the range of 0.35 mL/min to 0.55 mL/min (0.45 mL/min ± 0.1 mL/min), determining their influence on the retention times and resolution when compared with the original method conditions.

Variation of flow rate	0.45 mL/min (method conditions)		0.35 mL/min		0.55 mL/min	
	rt (min)	Resol.	rt (min)	Resol.	rt (min)	Resol.
Alfa-Ketoisoleucine	20.810	3.233	26.834	3.354	17.094	2.975
Alfa-Ketoleucine	24.323	1.649	31.289	1.667	19.915	1.478
Alfa-Ketovaline	15.260	6.504	19.682	6.789	12.555	6.106
Alfa-Ketophenylalanine	31.230	3.362	40.205	3.695	25.568	3.251
Alfa-Hydroxymethionine	26.186	3.362	33.597	3.695	21.355	3.251

3.2.P.5.3 Validation Report for Quantitative Determination of Keto and Hydroxy Acids in Ketosteril Film-Coated Tablets by High Performance Liquid Chromatography (HPLC)

Variation of flow rate	0.45 mL/min (method conditions)		0.35 mL/min		0.55 mL/min	
	rt (min)	Resol.	rt (min)	Resol.	rt (min)	Resol.
Comment	-------		Not critical: increase of retention times and slight increase of resolution.		Not critical: decrease of retention times and slight decrease of resolution, nevertheless the acceptance criteria (not less than 1) is fulfilled.	

3.3.6.4. Variation of column batch

The column batch was changed, determining their influence on the retention times and resolution when compared with the original method conditions.

Variation of column batch	Column internal nº 0164/16		Column internal nº 0162/16	
	rt (min)	Resol.	rt (min)	Resol.
Alfa-Ketoisoleucine	20.810	3.233	21.318	3.357
Alfa-Ketoleucine	24.323	1.649	24.949	1.828
Alfa-Ketovaline	15.260	6.504	15.586	6.676
Alfa-Ketophenylalanine	31.230	3.362	32.173	3.392
Alfa-Hydroxymethionine	26.186	3.362	27.013	3.392
Comment	-------		Not critical: slight increase of retention times and resolution.	

3.3.6.5. Variation of column temperature

The column temperature was changed in the range of 15ºC to 25ºC, determining their influence on the retention times and resolution when compared with the original method conditions.

Variation of column temperature	Room temperature (method conditions)		15ºC		25ºC	
	rt (min)	Resol.	rt (min)	Resol.	rt (min)	Resol.
Alfa-Ketoisoleucine	20.810	3.233	20.206	2.562	20.625	2.405
Alfa-Ketoleucine	24.323	1.649	24.491	2.130	24.189	1.364
Alfa-Ketovaline	15.260	6.504	14.497	4.645	15.081	4.810
Alfa-Ketophenylalanine	31.230	3.362	31.391	1.381	31.068	2.371
Alfa-Hydroxymethionine	26.186	3.362	28.278	1.381	26.295	2.371

Variation of column temperature	Room temperature (method conditions)		15ºC		25ºC	
	rt (min)	Resol.	rt (min)	Resol.	rt (min)	Resol.
Comment	-------		Not critical: slight variations on retention times and resolution, nevertheless the acceptance criteria (not less than 1) is fulfilled.		Not critical: slight variations on retention times and decrease of resolution nevertheless the acceptance criteria (not less than 1) is fulfilled.	

3.4. RESULTS ANALYSIS

3.4.1. Keto and Hydroxy acids assay

3.4.1.1. Alfa-Ketoisoleucine

The method linearity was demonstrated using the analyte as a single sample component and in the sample matrix in the range of 56.8 to 170.3%. The statistical analysis demonstrates the regression lines equivalency, proving that the sample matrix do not interfere in the alfa-ketoisoleucine quantification.
The mean recovery percentage obtained in the accuracy test is 98.0%, 97.3% and 99.2% for the level 80, 100 and 120% respectively.
Regarding the intermediate precision test, the mean and the RSD between the results of the two analysts are respectively 100.4% and 1.0%. The mean results are not statistically different.

3.4.1.2. Alfa-Ketoleucine

The method linearity was demonstrated using the analyte as a single sample component and in the sample matrix in the range of 55.5 to 166.4%. The statistical analysis demonstrates the regression lines equivalency, proving that the sample matrix do not interfere in the alfa-ketoleucine quantification.
The mean recovery percentage obtained in the accuracy test is 99.6%, 100.2% and 99.6% for the level 80, 100 and 120% respectively.
Regarding the intermediate precision test, the mean and the RSD between the results of the two analysts are respectively 102.1% and 0.4%. The mean results are not statistically different.

3.4.1.3. Alfa-Ketovaline

The method linearity was demonstrated using the analyte as a single sample component and in the sample matrix in the range of 55.8 to 167.4%. The statistical analysis demonstrates the regression lines equivalency, proving that the sample matrix do not interfere in the alfa-ketovaline quantification.
The mean recovery percentage obtained in the accuracy test is 99.5%, 99.2% and 100.0% for the level 80, 100 and 120% respectively.
Regarding the intermediate precision test, the mean and the RSD between the results of the two analysts are respectively 99.7% and 1.1%. The mean results are not statistically different.

3.4.1.4. Alfa-Ketophenylalanine

The method linearity was demonstrated using the analyte as a single sample component and in the sample matrix in the range of 53.0 to 159.0%. The statistical analysis demonstrates the regression lines equivalency, proving that the sample matrix do not interfere in the alfa-ketophenylalanine quantification.
The mean recovery percentage obtained in the accuracy test is 98.2%, 99.4% and 97.5% for the level 80, 100 and 120% respectively.
Regarding the intermediate precision test, the mean and the RSD between the results of the two analysts are respectively 101.6% and 1.2%. The mean results are not statistically different.

3.4.1.5. Alfa-Hydroxymethionine

The method linearity was demonstrated using the analyte as a single sample component and in the sample matrix in the range of 50.9 to 152.6%. The statistical analysis demonstrates the regression lines equivalency, proving that the sample matrix do not interfere in the alfa-hydroxymethionine quantification.
The mean recovery percentage obtained in the accuracy test is 97.9%, 98.0% and 98.9% for the level 80, 100 and 120% respectively.
Regarding the intermediate precision test, the mean and the RSD between the results of the two analysts are respectively 101.4% and 1.0%. The mean results are not statistically different.

3.4.2. Robustness

The sample and standard solutions are stable during 48 hours after their preparation.
The variations tested in the mobile phase composition, flow rate, column batch and column temperature demonstrate that they are not critical when compared with the method conditions, proving the method robustness.

3.4.3. Conclusion

The method is selective, linear, precise, accurate and robust, therefore valid to perform the quantitative determination of Keto and Hydroxy Acids in Ketosteril Drug-Coated Tablets.