3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

1. OBJECTIVE

Present the results obtained in the validation of the analytical method for assay of Amino acids in Ketosteril drug-coated tablets, by UPLC using UV detection.

2. DEFINITIONS AND ABBREVIATIONS

UPLC – Ultra Performance Liquid Chromatography
RSD – Relative Standard Deviation
rt – Retention time
N – Theoretical plates
Resol. – Resolution

3. RESULTS

3.1. Formula description

3.1.1. Active Substance

Amino acids, keto acids and hydroxy 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

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Components	Amount per unit (mg)	Function
L-Tryptophan	23,0	Active ingredient
L-Histidine	38,0	Active ingredient
L-Tyrosine	30,0	Active ingredient
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

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Components	Amount per unit (mg)	Function
Total		≈ 25

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

3.2. Method

3.2.1. Reagents and materials

AccQ-Tag Ultra Eluent A (Waters, batch: 0146153231, validity: 30 Nov. 2018)
AccQ-Tag Ultra Eluent B (Waters, batch: 0146142931, validity: 31 Oct. 2018)
AccQ-Tag Ultra Reagent Kit (Waters, batch: 8218853491, validity: 28 Feb. 2017) composed by vials of AccQ-Tag Ultra Borate Buffer, AccQ-Tag Ultra Reagent Powder and AccQ-Tag Ultra Reagent Diluent
0.02M Hydrochloride acid (logbook RU2, 50/2016, page 19)
MilliQ water, obtained from a Millipore System
L-Lysine acetate primary standard (ChromaDex, batch: 00012601-236, validity: 04 Aug. 2017, activity: 107.2%)
L-Threonine primary standard (ChromaDex, batch: 00020261-55V, validity: 04 Aug. 2017, activity: 99.5%)
L-Histidine primary standard (LGC Standards, batch: 66797, validity: 31 May 2017, activity: 99.9%)
L-Tyrosine primary standard (LGC Standards, batch: 38896, validity: 18 May 2017, activity: 99.9%)
L-Tryptophan primary standard (LGC Standards, batch: 34753, validity: 16 May 2017, activity: 99.7%)
Povidone (Batch: 160300498, validity: 30 June 2018)
Macrogol 6000 (Batch: 160100494, validity: 16 July 2019)
Alfa-Ketoisleucine calcium (Batch: 160400155, validity: 30 Nov. 2018)
Alfa-Ketoleucine calcium (Batch: 150300321, validity: 31 May. 2017)
Alfa-Ketovaline calcium (Batch: 160600190, validity: 28 Feb. 2018)
Alfa-Ketophenylalanine calcium (Batch: 150900484, validity: 30 June. 2018)
Alfa-Hydroxymethionine calcium (Batch: 160500145, validity: 31 Oct. 2018)
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 and pipettes

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3.2.2. Equipment

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
5120-II-0372: Water bath
2951-NI-7004: Calibrated thermometer
5120-II-0316: Micropipette 100-1000 µL
2951-NI-1035: Micropipette 10-100 µL

3.2.3. Standard stock solution: High range linearity – amino acids reference standards

It was weighed 105.0 mg of L-Lysine acetate, 53.1 mg of L-Threonine, 38.1 mg of L-Histidine, 30.1 mg of L-Tyrosine and 23.0 mg of L-Tryptophan in to a 100 mL volumetric flask. The volume was filled up with 0.02M Hydrochloride acid.

3.2.4. Standard working solution: High range linearity– amino acids reference standards

Standard working solutions were prepared according to table 1.

Level (%)	Dilution (mL standard stock solution → mL)
50	2 → 20
80	4 → 25
90	1.8 → 10
100	5 → 25
110	2.2 → 10
120	2.4 → 10
150	3 → 10

Table 1

The volume of each volumetric flask was filled up with 0.02M Hydrochloride acid.

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

It was weighed 105.1 mg of L-Lysine acetate, 52.9 mg of L-Threonine, 38.0 mg of L-Histidine, 30.0 mg of L-Tyrosine and 22.9 mg of L-Tryptophan in to a 100 mL volumetric flask. The volume was filled up with 0.02M Hydrochloride acid.

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 → 50
80	8 → 50

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

Table 2

To each 50 mL volumetric flask was added placebo amounts corresponding to the 100% composition and the volume were filled up with 0.02M Hydrochloride acid.

3.2.7. Standard solution

It was weighed about 150.0 mg of L-Lysine acetate, 53.0 mg of L-Threonine, 38.0 mg of L-Histidine, 30.0 mg of L-Tyrosine and 23.0 mg of L-Tryptophan in to a 100 mL volumetric flask. The volume was filled up with 0.02M Hydrochloride acid.
Finally, 5 mL of the previous solution were diluted to 25 mL with 0.02M Hydrochloride acid.

3.2.8. Test solution

It was weighed 10 tablets of Ketosteril and transferred to a 1000 mL volumetric flask, 0.02M Hydrochloride acid was added and the solution was stirred until tablets complete dissolution. 5 mL of the previous solution were diluted to 25 mL with 0.02M Hydrochloride acid.

3.2.9. AccQ-Tag Ultra Reagent

It was transferred 1 mL of AccQ-Tag Ultra Reagent Diluent to a AccQ-Tag Ultra Reagent Powder vial, previously tapped. The vial was mixed for 10 seconds and heated at 55ºC in a water bath until complete dissolution of the powder.

3.2.10. Standard and Test solution derivatization

It was pipetted 40 µL of standard/ test solution, 280 µL of AccQ-Tag Ultra Borate Buffer and 80 µL of AccQ-Tag Ultra Reagent into a total recovery vial. The vial was mixed, incubated for 1 minute at room temperature and heated to 55ºC in a water bath for 10 minutes.

3.2.11. Chromatographic conditions

- Column: Waters, AccQ-Tag Ultra C18, 100x2.1 mm, 1.7 µm, Part number: 186003837, Serial number: 02403433316092, Internal number: 0175/16
- Column: Waters, AccQ-Tag Ultra C18, 100x2.1 mm, 1.7 µm, Part number: 186003837, Serial number: 02403523015810, Internal number: 0163/16 (Robustness - variation of column batch)
- λ= 260 nm
- Mobile phase A: Mixture of 50 mL of AccQ-Tag Ultra Eluent A with 950 mL of water
- Mobile phase A: Mixture of 69 mL of AccQ-Tag Ultra Eluent A with 931 mL of water (Robustness - variation of the water proportion in mobile phase A)

- Mobile phase A: Mixture of 31 mL of AccQ-Tag Ultra Eluent A with 969 mL of water (Robustness - variation of the water proportion in mobile phase A)
- Mobile phase B: AccQ-Tag Ultra Eluent B
- Flow rate: 0.7 mL/min
- Flow rate: 0.6 mL/min and 0.8 mL/min (Robustness - variation of the flow rate)
- Column temperature: 55ºC
- Column temperature: 50ºC and 60ºC (Robustness - variation of the column temperature)
- Sample tray temperature: 20ºC
- Injection volume: 1 µL
- Gradient time table:

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.00	99.9	0.1
0.54	99.9	0.1
5.74	90.9	9.1
7.74	78.8	21.2
8.04	40.4	59.6
8.50	40.4	59.6
8.60	10.0	90.0
9.00	10.0	90.0
9.10	99.9	0.1
10.0	99.9	0.1

3.3. Validation Parameters

3.3.1. System Suitability

Acceptance criteria:
- The RSD between 5 injections should not be more than 3.66%
- The number of theoretical plates should not be less than 2005
- The resolution between all the 5 peaks should not be less than 1.5

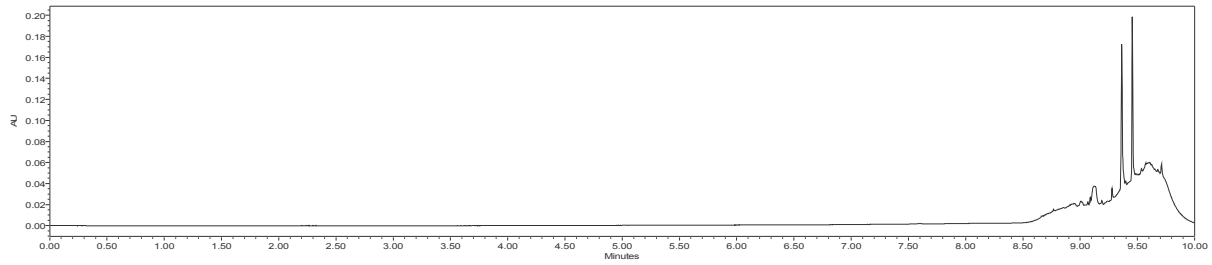
Substance	RSD	Plates nº	Resolution
L-Lysine acetate	0.08	1770587	10.945
L-Threonine	0.12	401234	48.405
L-Histidine	0.26	9712	28.409
L-Tyrosine	0.21	1441871	56.777
L-Tryptophan	0.09	10196398	56.777

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

3.3.2. Selectivity

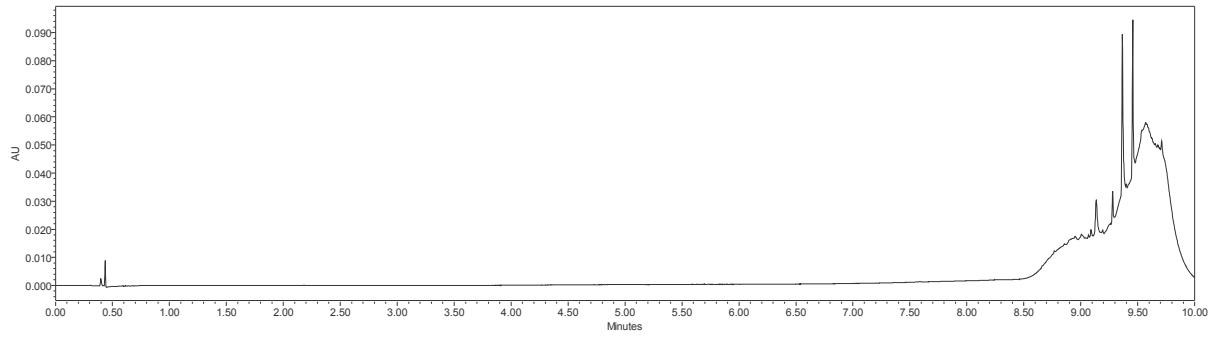
The placebo solution doesn't interfere in the Amino acids quantification.

3.3.2.1. Mobile Phase A



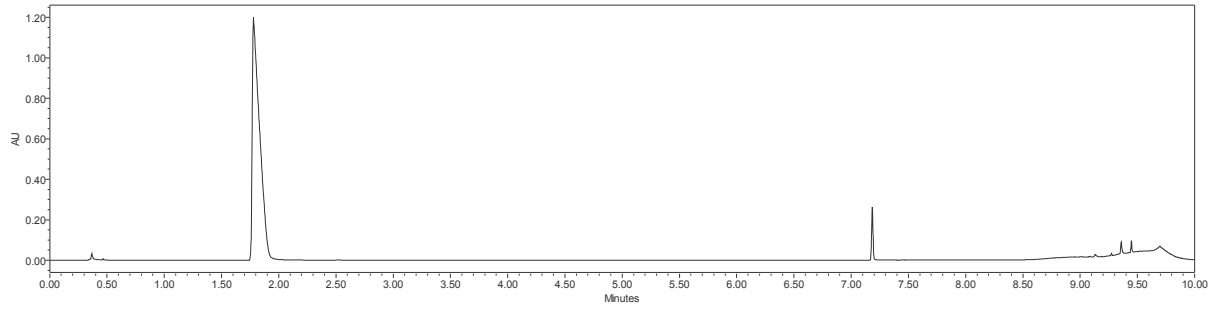
Chromatogram 1

3.3.2.2. Mobile Phase B



Chromatogram 2

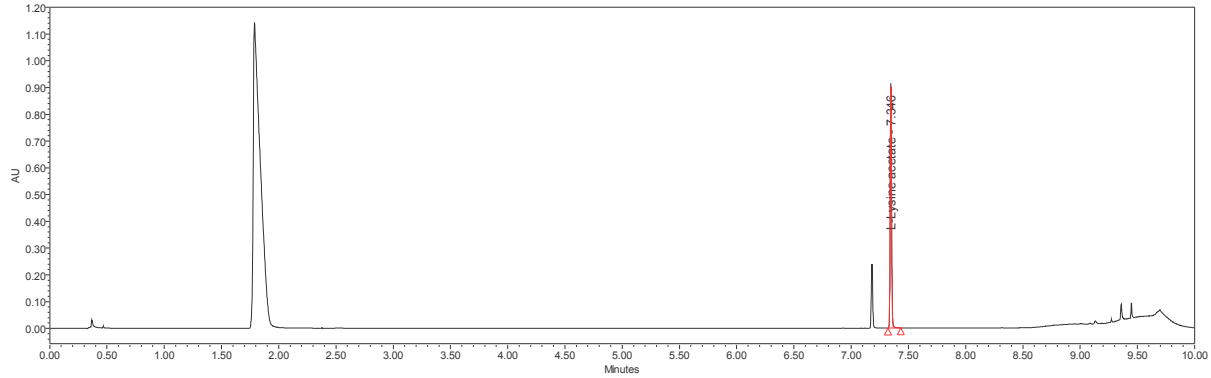
3.3.2.3. Solvent



Chromatogram 3

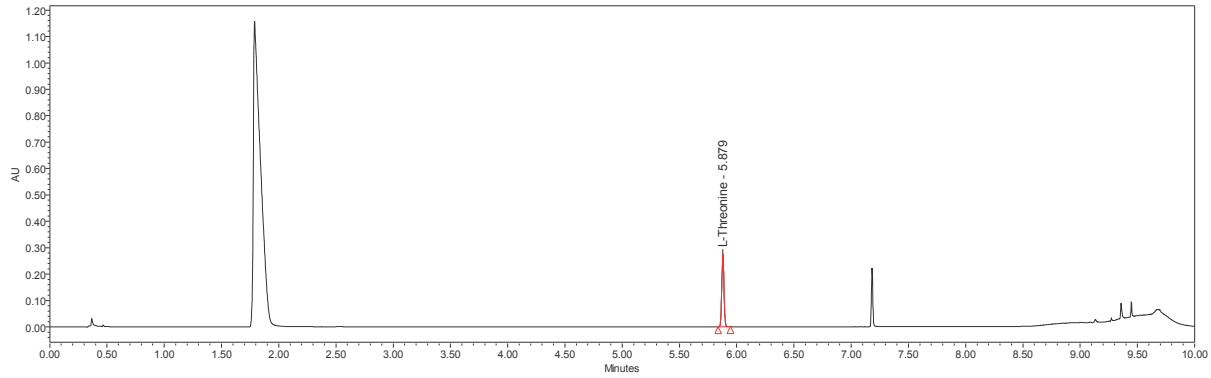
3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

3.3.2.4. L-Lysine acetate standard solution



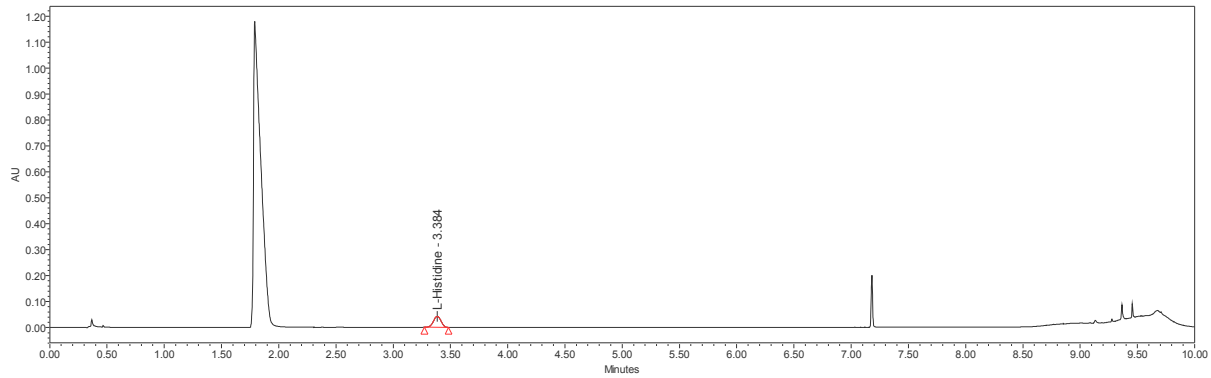
Chromatogram 4

3.3.2.5. L-Threonine standard solution



Chromatogram 5

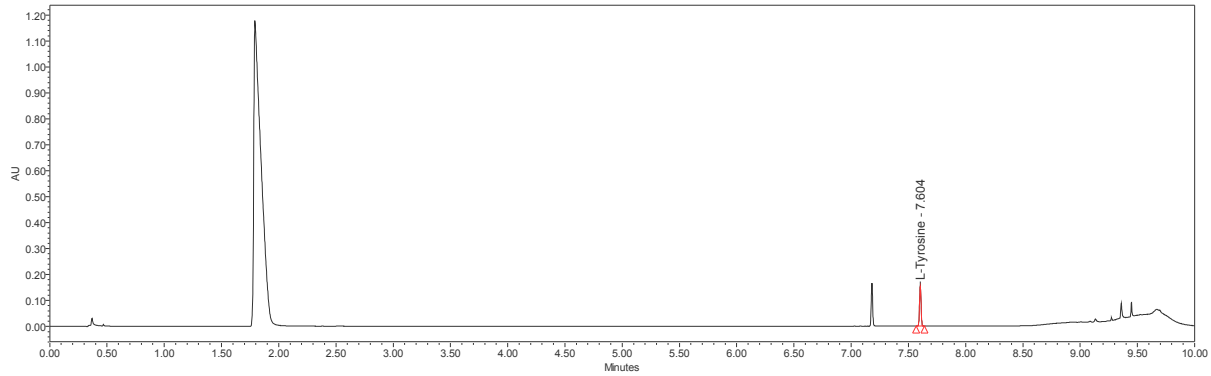
3.3.2.6. L-Histidine standard solution



Chromatogram 6

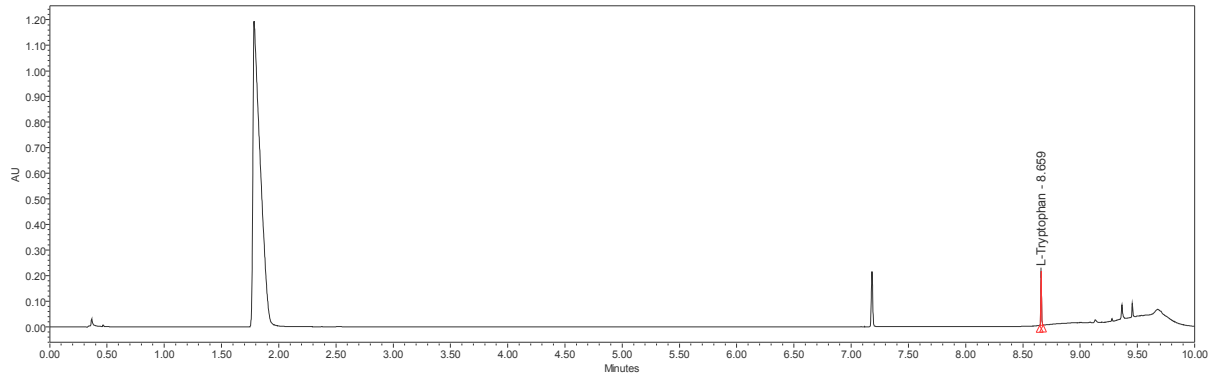
3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

3.3.2.7. L-Tyrosine standard solution



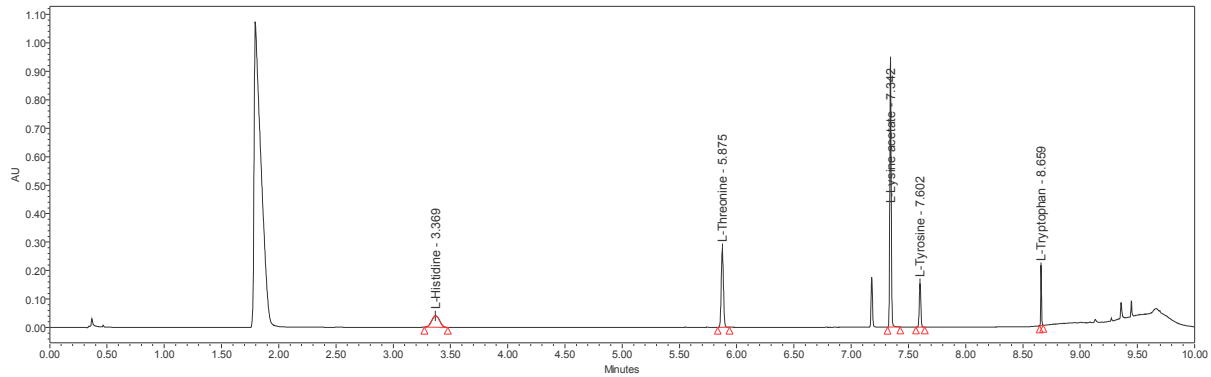
Chromatogram 7

3.3.2.8. L-Tryptophan standard solution



Chromatogram 8

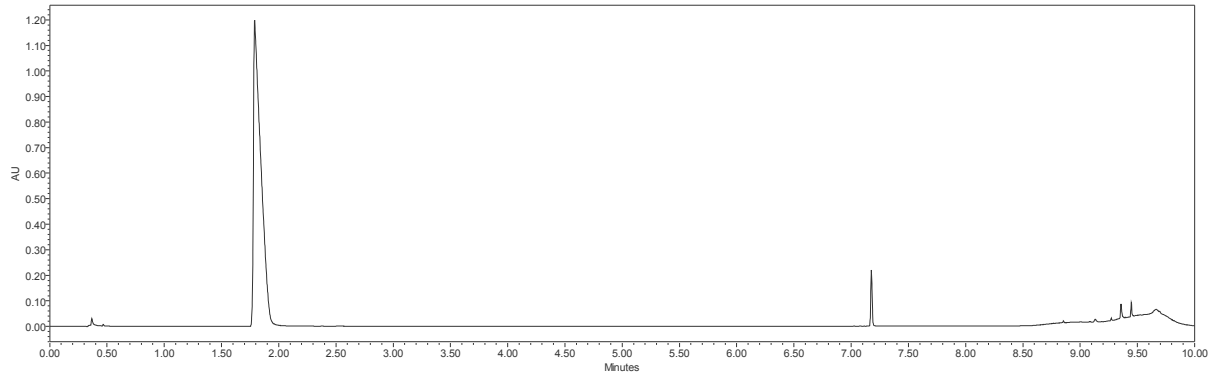
3.3.2.9. Standard solution



Chromatogram 9

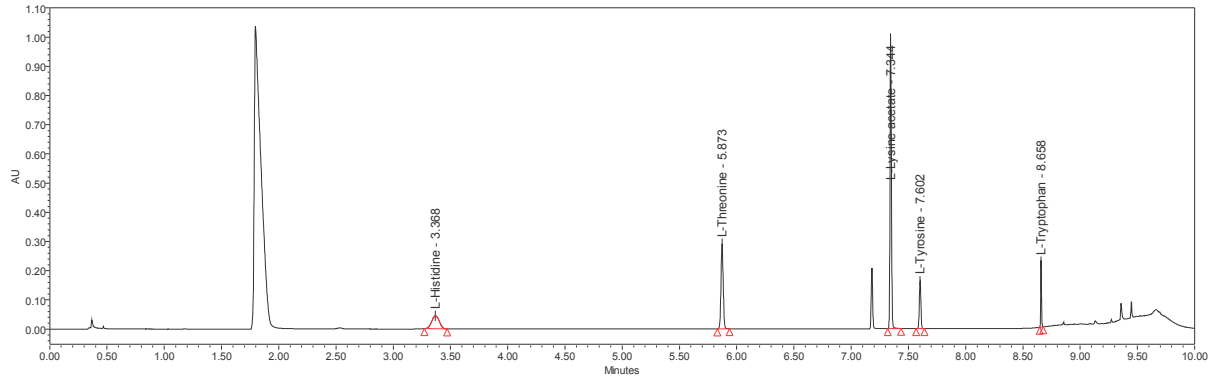
3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

3.3.2.10. Placebo solution



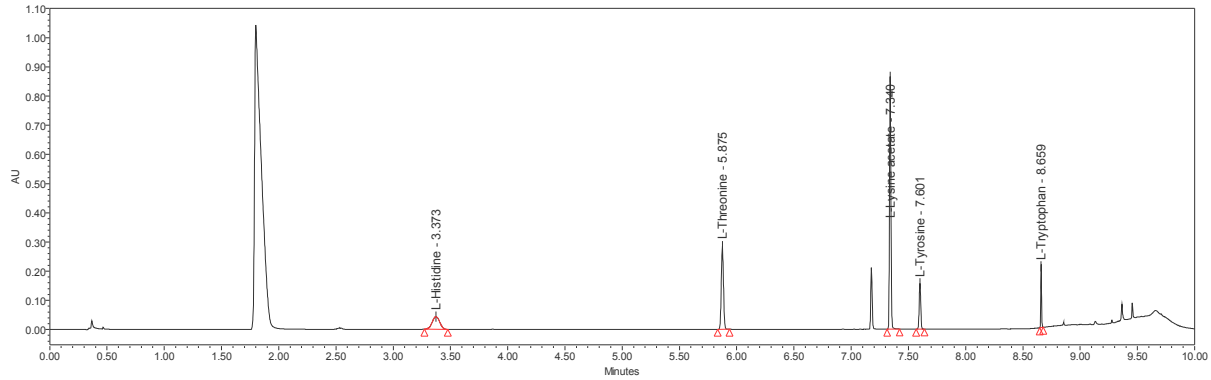
Chromatogram 10

3.3.2.11. Placebo + standard solution



Chromatogram 11

3.3.2.12. Test solution



Chromatogram 12

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

3.3.3. High range linearity

a) Amino acids 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.

- L-Lysine acetate

Level (%)	L-Lysine acetate concentration (%)	Area
50	53.600	400672
		399999
		400722
	Mean	400464
	RSD (%)	0.1
80	85.760	616864
		618338
		619290
	Mean	618164
	RSD (%)	0.2
90	96.480	731545
		733041
		732220
	Mean	732269
	RSD (%)	0.1
100	107.200	833652
		834320
		848556
	Mean	838843
	RSD (%)	1.0
110	117.920	900630
		889145
		890155
	Mean	893310
	RSD (%)	0.7

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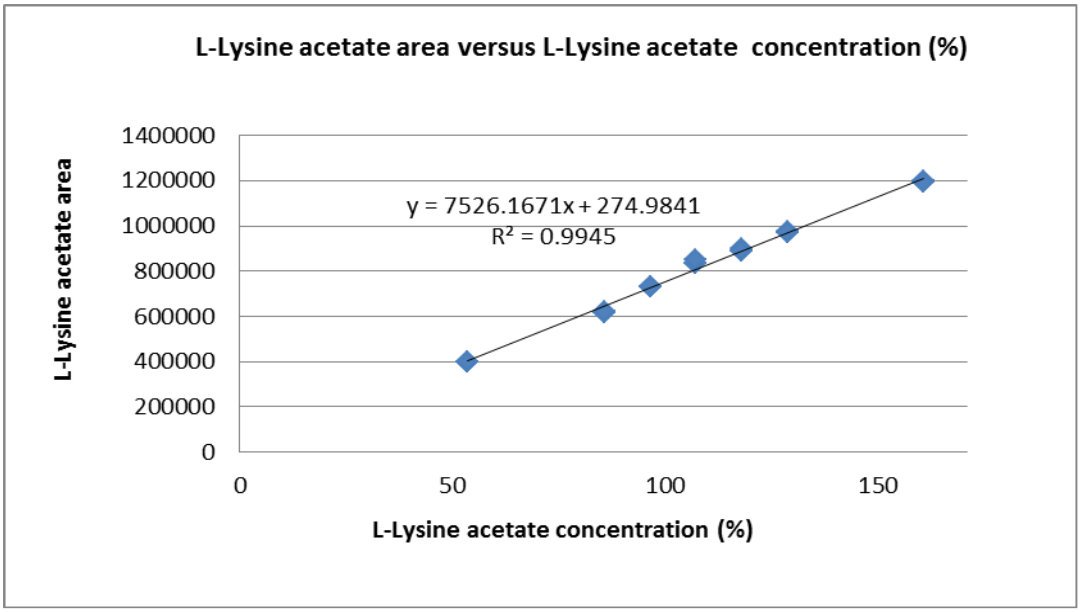
Level (%)	L-Lysine acetate concentration (%)	Area
120	128.640	974853
		969889
		969374
	Mean	971372
	RSD (%)	0.3
150	160.800	1196407
		1194458
		1194552
	Mean	1195139
	RSD (%)	0.1



Linear regression equation (y=7526.1671x + 274.9841)

m= 7526.1671
b= 274.9841
r= 0.9972

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- L-Threonine

Level (%)	L-Threonine concentration (%)	Area
50	49.844	183068
		182677
		183008
	Mean	182918
	RSD (%)	0.1
80	79.750	282376
		282845
		283049
	Mean	282757
	RSD (%)	0.1
90	89.719	335462
		336302
		335883
	Mean	335882
	RSD (%)	0.1
100	99.688	384141
		384552
		391247
	Mean	386647
	RSD (%)	1.0
110	109.657	413842
		408298
		408050
	Mean	410063
	RSD (%)	0.8
120	119.625	447201
		444962
		444266
	Mean	445476
	RSD (%)	0.3
150	149.532	548465

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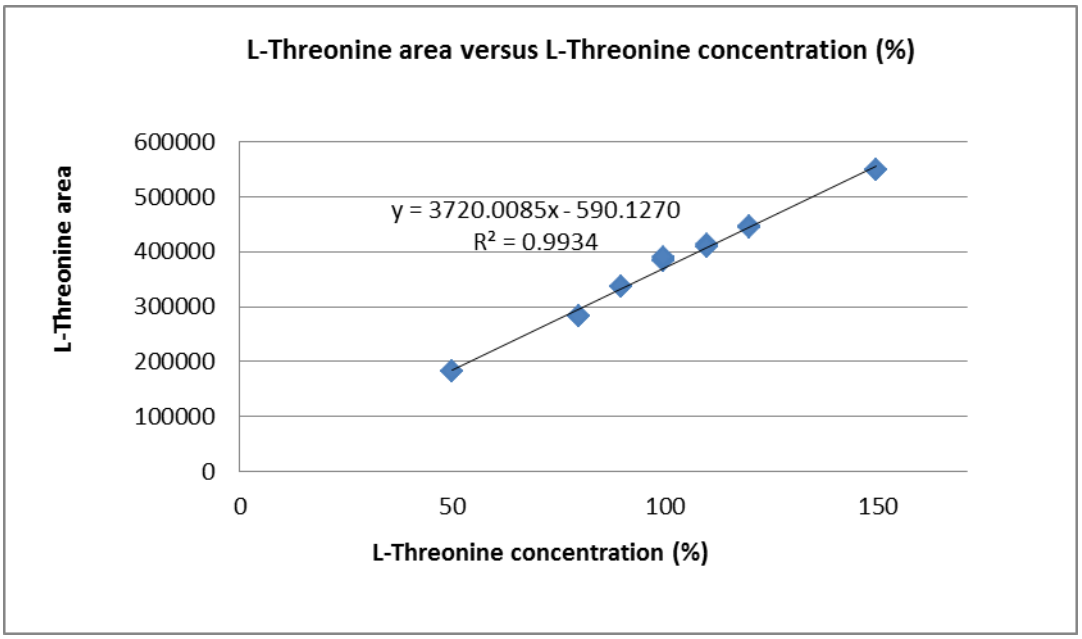
Level (%)	L-Threonine concentration (%)	Area
		547790
		547747
	Mean	548000
	RSD (%)	0.1



Linear regression equation (y=3720.0085x – 590.1270)

m= 3720.0085
b= -590.1270
r= 0.9967

- <u>L-Histidine</u>

Level (%)	L-Histidine concentration (%)	Area
50	50.081	100272
		99949
		99962
	Mean	100061
	RSD (%)	0.2
80	80.130	154786
		154952
		155386

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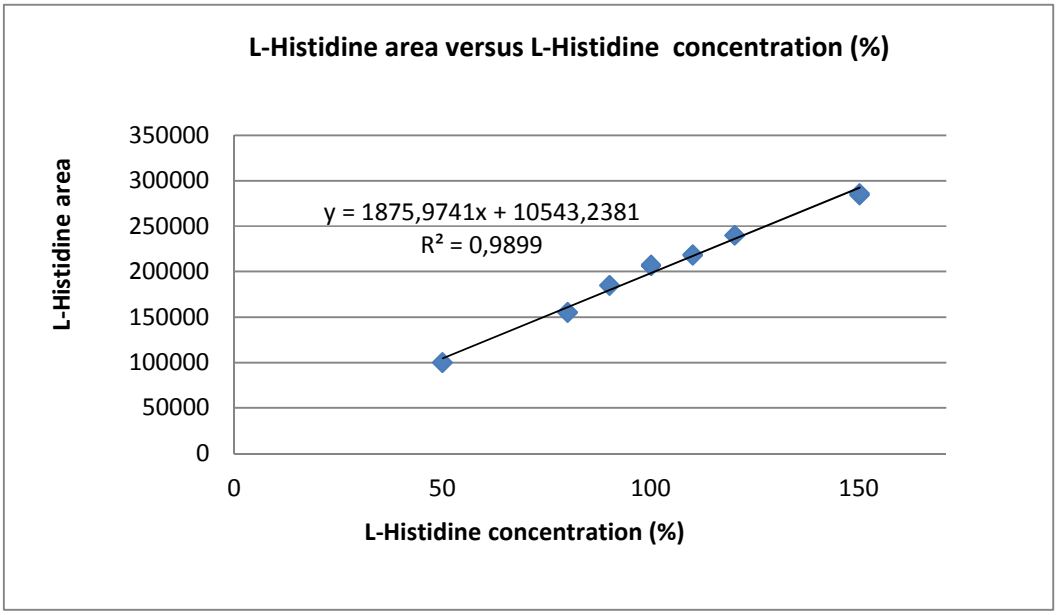
Level (%)	L-Histidine concentration (%)	Area
	Mean	155041
	RSD (%)	0.2
90	90.147	184557
		184403
		184717
	Mean	184559
	RSD (%)	0.1
100	100.163	205861
		206377
		207481
	Mean	206573
	RSD (%)	0.4
110	110.179	218769
		218018
		217660
	Mean	218149
	RSD (%)	0.3
120	120.195	239939
		239716
		239831
	Mean	239829
	RSD (%)	0.1
150	150.244	285939
		284886
		283910
	Mean	284912
	RSD (%)	0.4

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)



Linear regression equation (y=1875.9741x + 10543.2381)

m= 1875.9741
b= 10543.2381
r= 0.9949

- L-Tyrosine

Level (%)	L-Tyrosine concentration (%)	Area
50	50.117	70058
		69976
		70184
	Mean	70073
	RSD (%)	0.2
80	80.186	108501
		108192
		108457
	Mean	108383
	RSD (%)	0.2
90	90.210	128191
		128868
		129025
	Mean	128694

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Level (%)	L-Tyrosine concentration (%)	Area
	RSD (%)	0.3
100	100.233	147663
		147854
		150264
	Mean	148594
	RSD (%)	1.0
110	110.256	158517
		156499
		156544
	Mean	157187
	RSD (%)	0.7
120	120.280	171525
		170696
		170427
	Mean	170882
	RSD (%)	0.3
150	150.350	210049
		209843
		209864
	Mean (%)	209919
	RSD (%)	0.1

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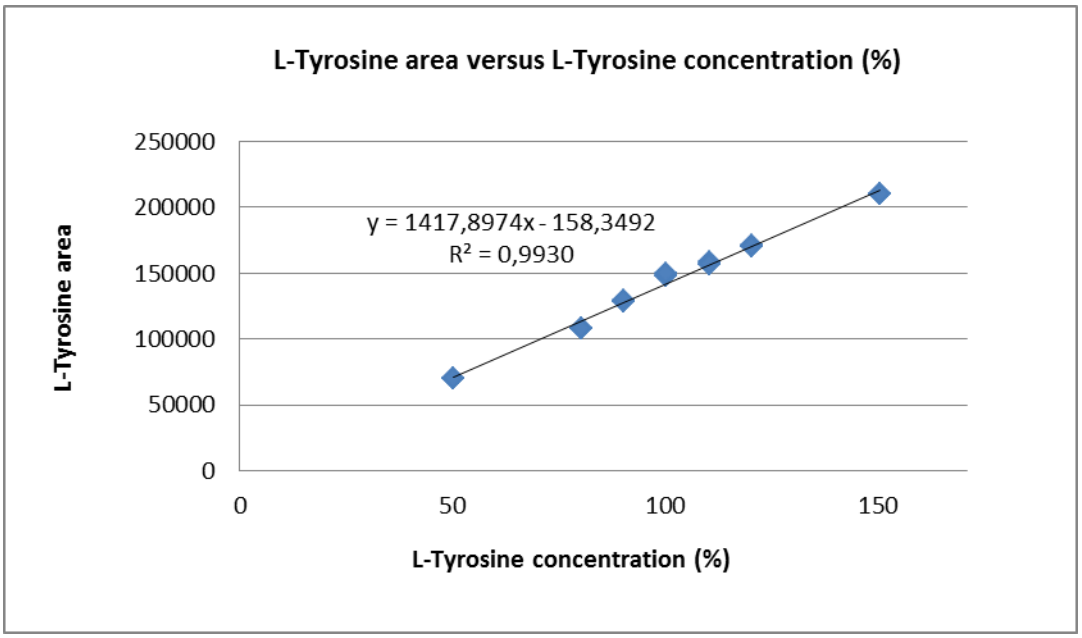

Linear regression equation (y=1417.8974x – 158.3492)

m= 1417.8974
b= -158.3492
r= 0.9965

- L-Tryptophan

Level (%)	L-Tryptophan concentration (%)	Area
50	49.850	47914
		47835
		47926
	Mean	47892
	RSD (%)	0.1
80	79.760	73750
		73860
		73936
	Mean	73849
	RSD (%)	0.1
90	89.730	87509
		87715
		87664
	Mean	87629

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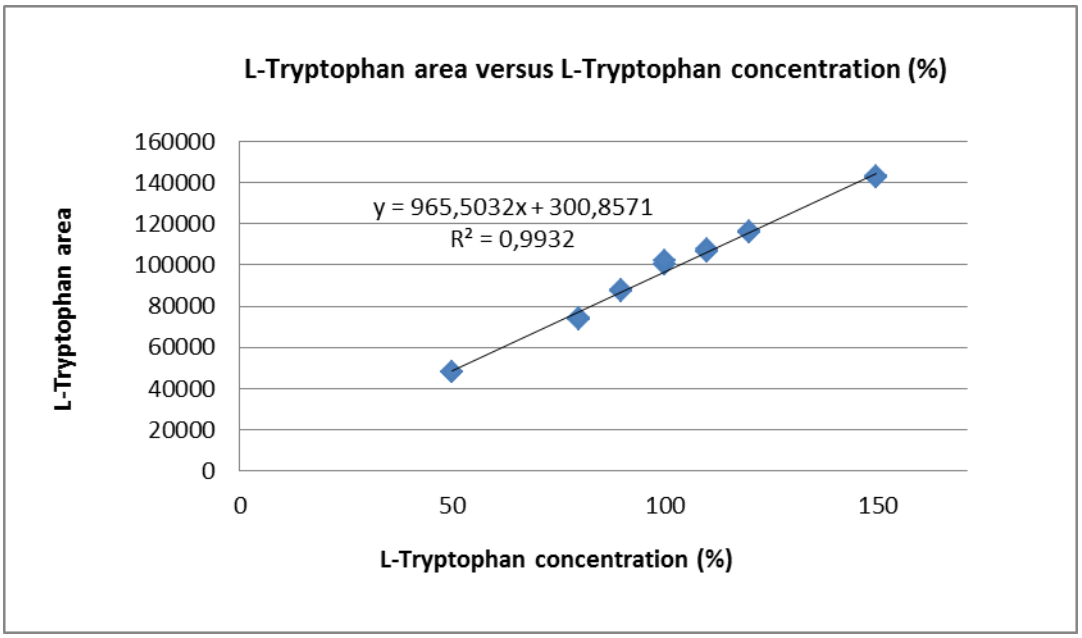
Level (%)	L-Tryptophan concentration (%)	Area
	RSD (%)	0.1
100	99.700	100321
		100413
		102201
	Mean	100978
	RSD (%)	1.1
110	109.670	107804
		106271
		106410
	Mean	106829
	RSD (%)	0.8
120	119.640	116514
		115938
		115799
	Mean	116084
	RSD (%)	0.3
150	149.550	142923
		142378
		142711
	Mean	142671
	RSD (%)	0.2

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)



Linear regression equation (y=965.5032x + 300.8571)

m= 965.5032
b= 300.8571
r= 0.9966

b) Matrix effect: placebo + amino acids 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.

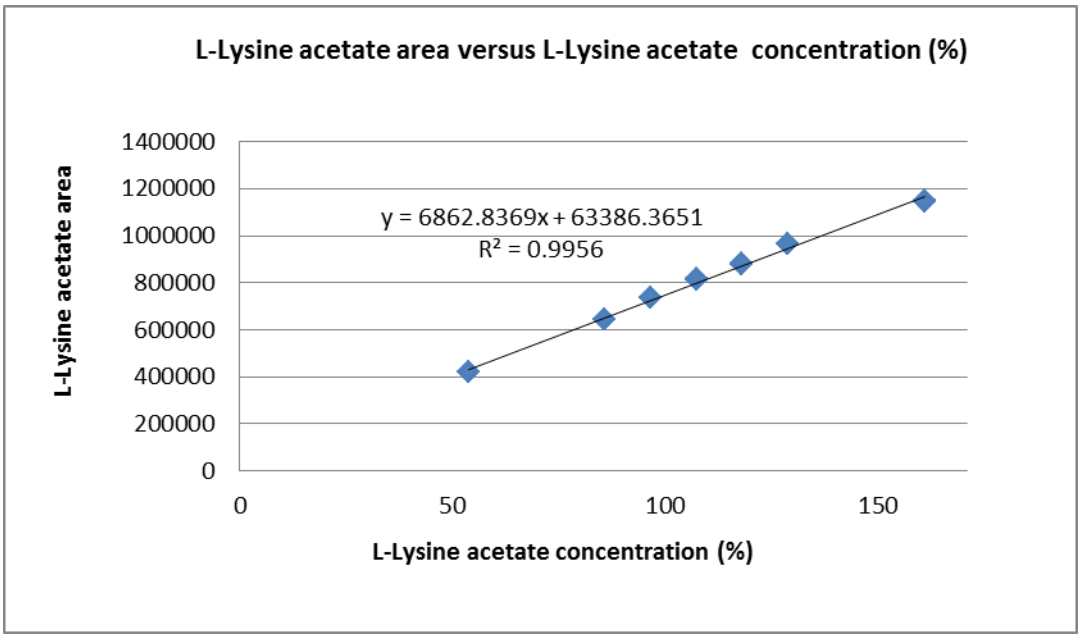
- L-Lysine acetate

Level (%)	L-Lysine acetate concentration (%)	Area
50	53.651	418173
		420014
		418319
	Mean	418835
	RSD (%)	0.2
80	85.842	641275
		642061
		641068
	Mean	641468

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Level (%)	L-Lysine acetate concentration (%)	Area
	RSD (%)	0.1
90	96.572	735445
		735740
		735970
	Mean	735718
	RSD (%)	0.0
100	107.302	813077
		814217
		815063
	Mean	814119
	RSD (%)	0.1
110	118.032	879403
		879697
		880495
	Mean	879865
	RSD (%)	0.1
120	128.763	963495
		963008
		964532
	Mean	963678
	RSD (%)	0.1
150	160.953	1142579
		1149799
		1142016
	Mean	1144798
	RSD (%)	0.4

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)



Linear regression equation (y=6862.8369 + 63386.3651)

m= 6862.8369
b= 63386.3651
r= 0.9978

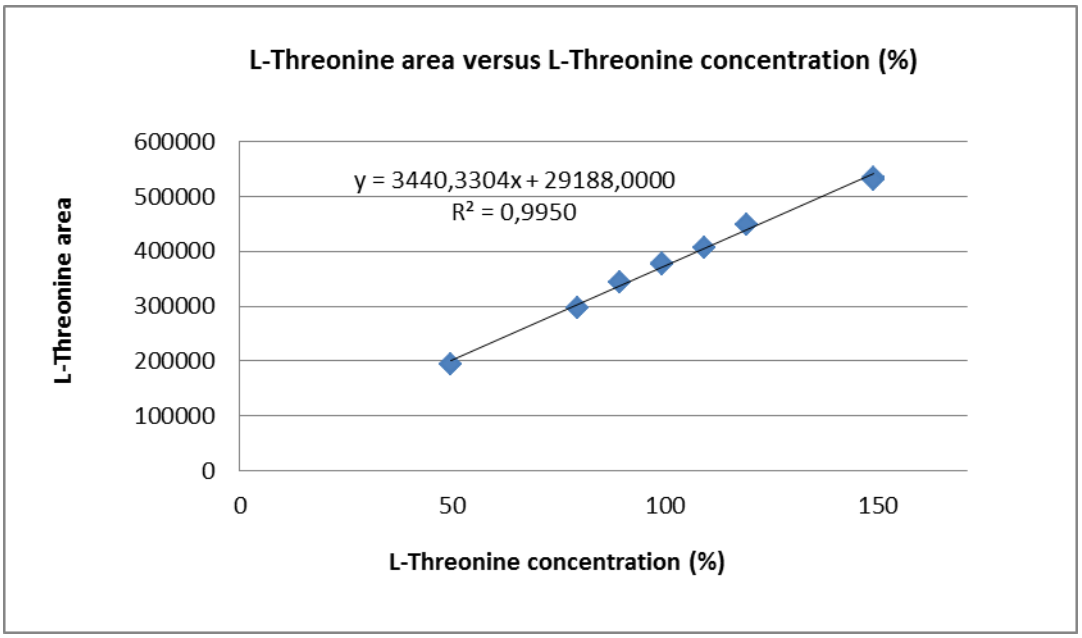
- L-Threonine

Level (%)	L-Threonine concentration (%)	Area
50	49.656	193933
		194464
		193454
	Mean	193950
	RSD (%)	0.3
80	79.450	296169
		296659
		296426
	Mean	296418
	RSD (%)	0.1
90	89.381	342996
		343072
		343641
	Mean	343237

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Level (%)	L-Threonine concentration (%)	Area
	RSD (%)	0.1
100	99.312	376570
		377220
		377258
	Mean	377016
	RSD (%)	0.1
110	109.243	406838
		407143
		407330
	Mean	407104
	RSD (%)	0.1
120	119.175	447674
		447328
		447579
	Mean	447527
	RSD (%)	0.0
150	148.968	529331
		533569
		529301
	Mean	530733
	RSD (%)	0.5

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)



Linear regression equation (y=3440.3304x + 29188.0000)

m= 3440.3304
b= 29188.0000
r= 0.9975

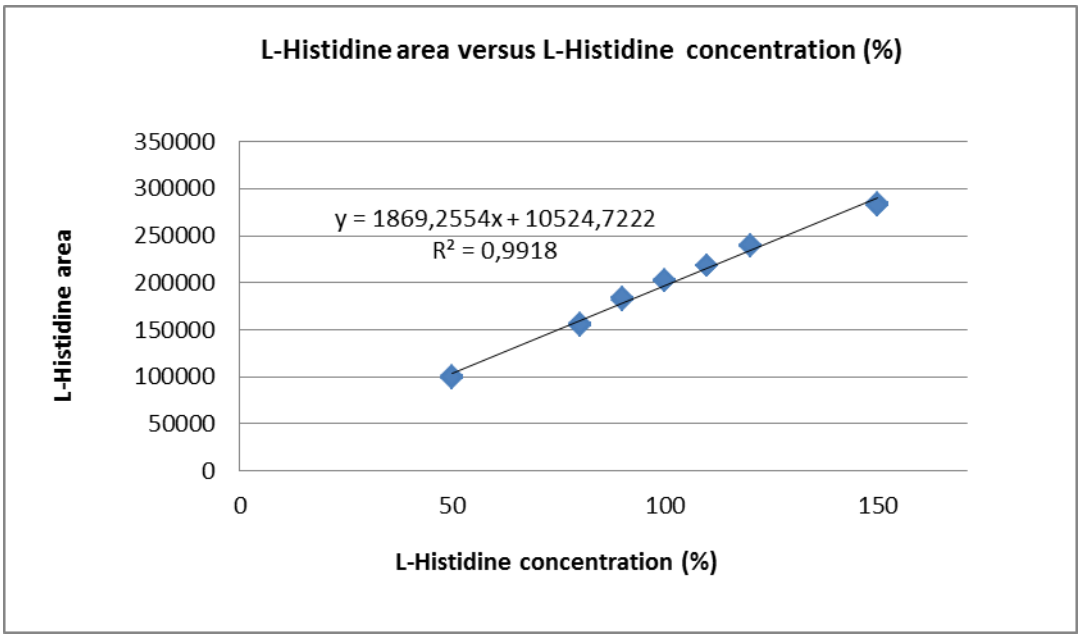
- L-Histidine

Level (%)	L-Histidine concentration (%)	Area
50	49.950	98264
		100471
		100160
	Mean	99632
	RSD (%)	1.2
80	79.920	154164
		156230
		155413
	Mean	155269
	RSD (%)	0.7
90	89.910	181670
		184196
		184032
	Mean	183299

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Level (%)	L-Histidine concentration (%)	Area
	RSD (%)	0.8
100	99.900	202309
		201907
		202506
	Mean	202241
	RSD (%)	0.2
110	109.890	217543
		217796
		218475
	Mean	217938
	RSD (%)	0.2
120	119.880	239297
		238798
		239739
	Mean	239278
	RSD (%)	0.2
150	149.850	282307
		284940
		282313
	Mean	283187
	RSD (%)	0.5

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)



Linear regression equation (y=1869.2554x + 10524.7222)

m= 1869.2554
b= 10524.722
r= 0.9959

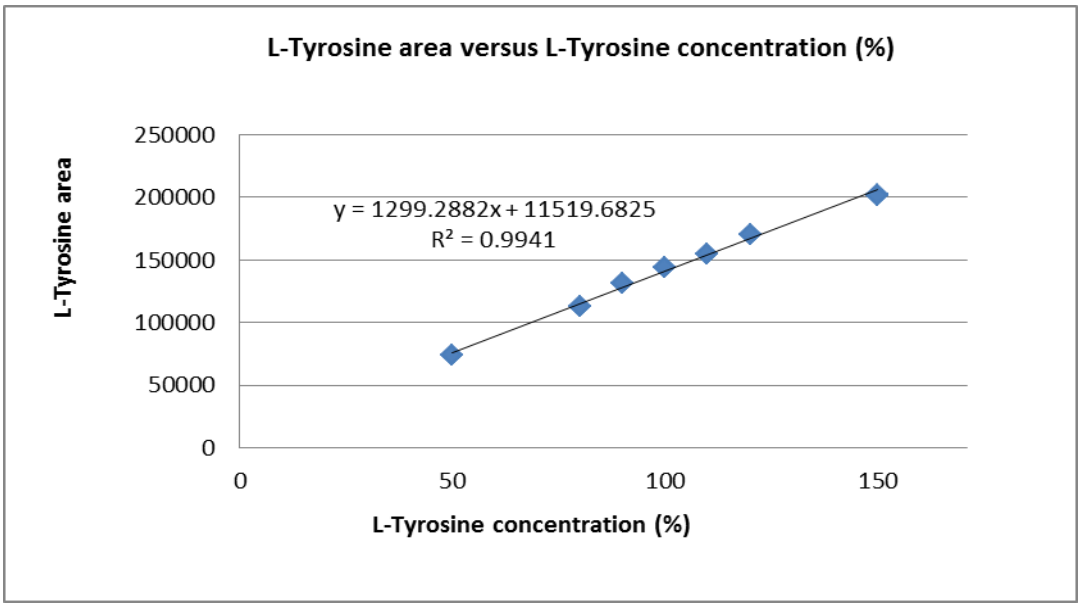
- L-Tyrosine

Level (%)	L-Tyrosine concentration (%)	Area
50	49.950	73680
		74102
		73827
	Mean	73870
	RSD (%)	0.3
80	79.920	112675
		112941
		112710
	Mean	112775
	RSD (%)	0.1
90	89.910	131310
		131338
		131437
	Mean	131362

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Level (%)	L-Tyrosine concentration (%)	Area
	RSD (%)	0.1
100	99.900	143779
		144029
		144247
	Mean	144018
	RSD (%)	0.2
110	109.890	154757
		154879
		155028
	Mean	154888
	RSD (%)	0.1
120	119.880	170404
		170340
		170675
	Mean	170473
	RSD (%)	0.1
150	149.850	201536
		202754
		201242
	Mean	201844
	RSD (%)	0.4

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)



Linear regression equation (y=1299.2882x + 11519.6825)

m= 1299.2882
b= 11519.6825
r= 0.9971

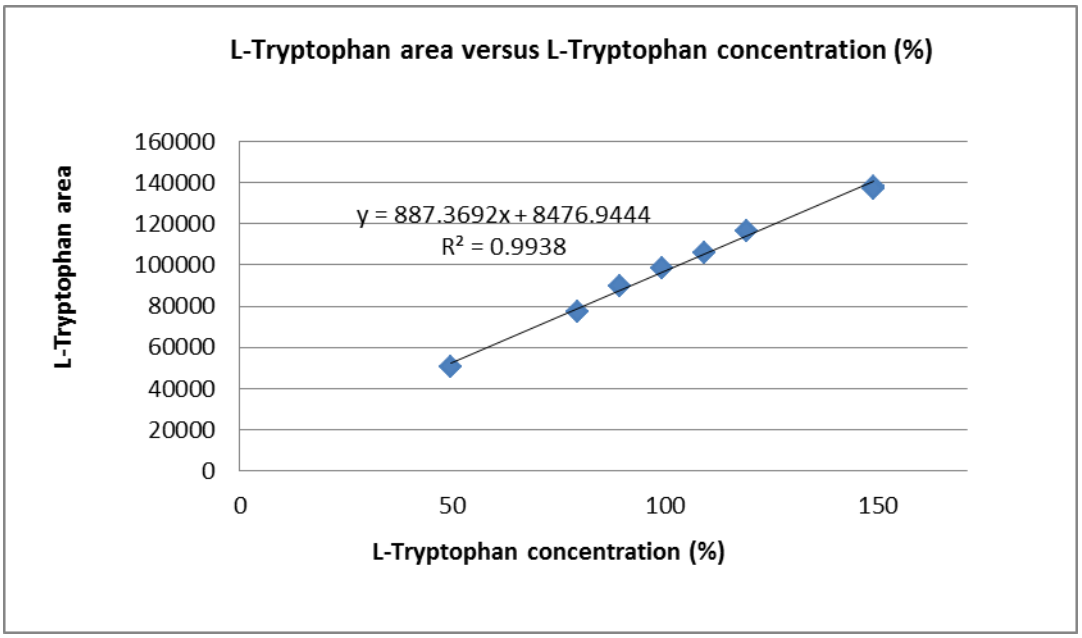
- L-Tryptophan

Level (%)	L-Tryptophan concentration (%)	Area
50	49.633	50624
		50820
		50672
	Mean	50705
	RSD (%)	0.2
80	79.413	77156
		77284
		77100
	Mean	77180
	RSD (%)	0.1
90	89.340	89850
		89853
		89936
	Mean	89880

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Level (%)	L-Tryptophan concentration (%)	Area
	RSD (%)	0.1
100	99.267	98387
		98541
		98647
	Mean	98525
	RSD (%)	0.1
110	109.193	105636
		105708
		105778
	Mean	105707
	RSD (%)	0.1
120	119.120	116364
		116266
		116513
	Mean	116381
	RSD (%)	0.1
150	148.900	201536
		202754
		201242
	Mean	201844
	RSD (%)	0.4

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)



Linear regression equation (y=887.3962x + 8476.9444)

m= 887.3692
b= 8476.9444
r= 0.9969

c) Statistical analysis

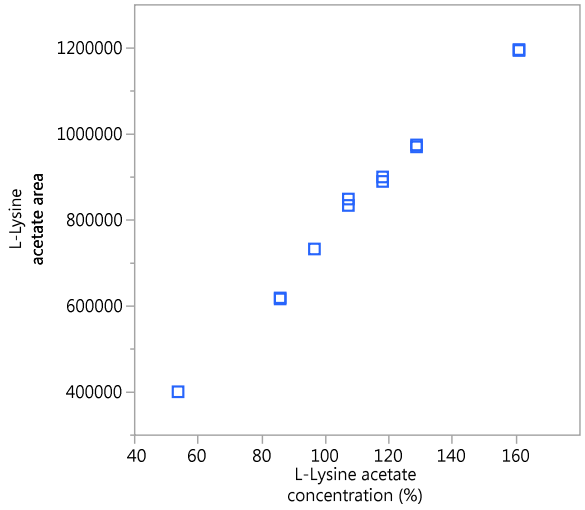
The statistical analysis was performed with assistance of the statistical software JMP Statistical Discovery from SAS, version 12 and XLSTAT 2016.

L-Lysine Acetate

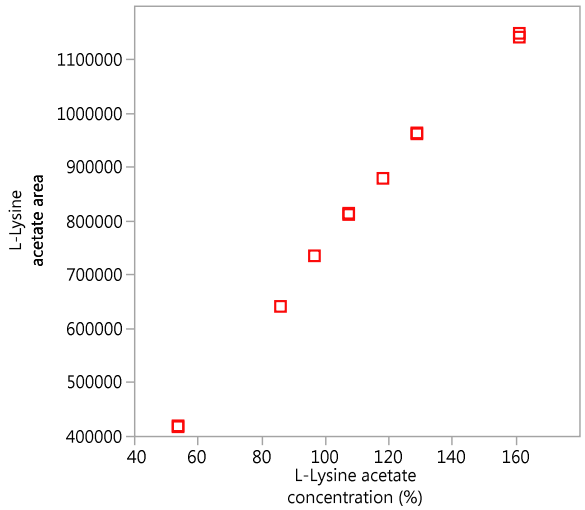
Scatterplots
In order to assess whether there is a linear relationship between the Concentration(%) and the Area of the analyte two scatterplots were constructed, one in which the analyte is the only component of the sample, and another in which the analyte is in a complex matrix with other constituents.
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,

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)



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=0.9972 and when is in a complex matrix with other constituents r=0.9978.

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	274,984	14376,981	0,019	0,985	-29816,382	30366,350
Conc. (%)	7526,167	128,711	58,473	0,000	7256,772	7795,562

In the second case,

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	63386,365	11756,899	5,391	0,000	38778,893	87993,838
Conc. (%)	6862,837	105,154	65,264	0,000	6642,747	7082,927

Therefore, we have:

$$a_1 = 274{,}984,\ b_1 = 7526{,}167 \qquad a_2 = 63386{,}365,\ b_2 = 6862{,}837$$

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 rejected, respectively, p=0.043 and p=0.000.
Therefore, it is concluded that the estimates found by the method of ordinary least squares are not valid. The following tables show test results:

- in the first case,

C (Observed value)	0,572
C (Critical value)	0,561
p-value (unilateral)	0,043
alfa	0,05

- In the second case,

C (Observed value)	0,851
C (Critical value)	0,561
p-value (unilateral)	0,000
alfa	0,05

Adjusting the linear regression lines using the Weighted Least Squares method

Using the Cochran´s C test it was concluded that heteroscedasticity exists in both cases, when the analyte is the only component of the sample and when is contained in a complex matrix, which led to the need for 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 of Intercepts, $a_1$and a_2, and Slopes, b_1 and b_2, are as follows:

- the analyte is the only component of the sample,

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	777,419	4686,242	0,166	0,870	-9030,997	10585,835
Conc. (%)	7463,750	57,800	129,130	0,000	7342,772	7584,727

- the analyte is contained in a complex matrix,

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	33247,079	10411,306	3,193	0,005	11455,965	55038,193
Conc. (%)	7227,698	104,233	69,341	0,000	7009,535	7445,862

In short,

$$a_1 = 777{,}419,\ b_1 = 7463{,}750 \qquad a_2 = 33247{,}079,\ b_2 = 7227{,}698$$

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. In both cases, ANOVA was conducted considering weighted least squares method.

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

For the analyte to be the only component we have:

ANOVA

	SS	*df*	*MS*	*F*	*p-value*
Regression	1244537445246	1	1244537445246	16674,47	0,000
Residual	1418108872	19	74637309		
Total	1245955554118	20			

Regression Statistics	
R Square	0,9989
Adjusted R Square	0,9988
Standard Error	8639,29
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 99.89% of the variability in the data.
In case the analyte is contained in a complex matrix,

ANOVA

	SS	*df*	*MS*	*F*	*p-value*
Regression	244632035757	1	244632035757	4808,235	0,000
Residual	966676762	19	50877724		
Total	245598712519	20			

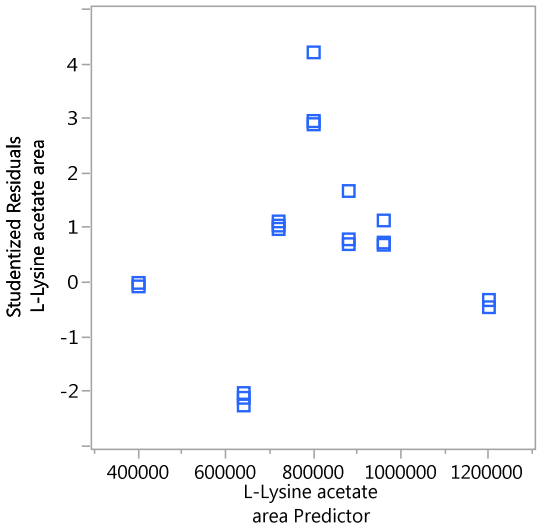
Regression Statistics	
R Square	0,9961
Adjusted R Square	0,9959
Standard Error	7132,86
Observations	21

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 99.61% of the variability in the data.

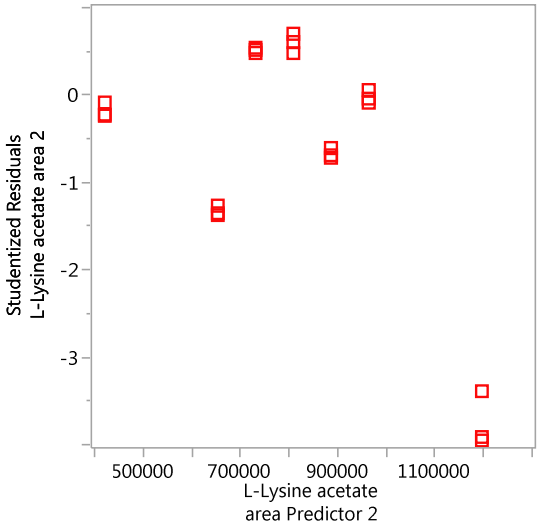
Residuals
Residuals are extremely useful to check whether a particular linear regression model is appropriate for the data. So a scatterplot must be built in which residuals and predictive values for Area are crossed. For the regression line to be appropriate is necessary that the scatterplot does not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

$$b_1 = 7463{,}750 \qquad b_2 = 7227{,}698$$

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

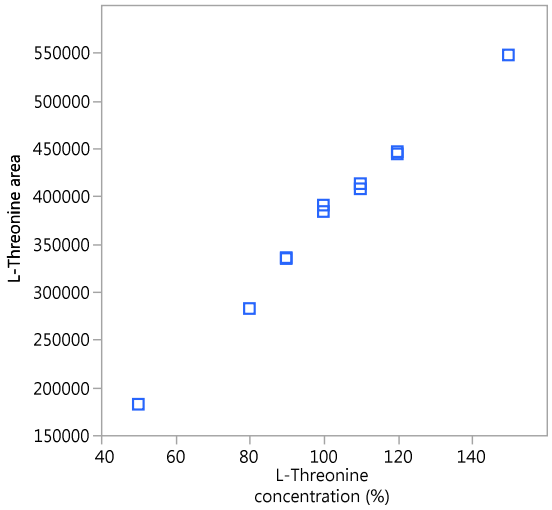
The following table indicates the test results,

Paralell Fit SSE	*Full SSE*	*NDF*	*DDF*	*F Ratio*	*Prob > F*
2599835094,40	2384318081	1	38	3,435	0,072

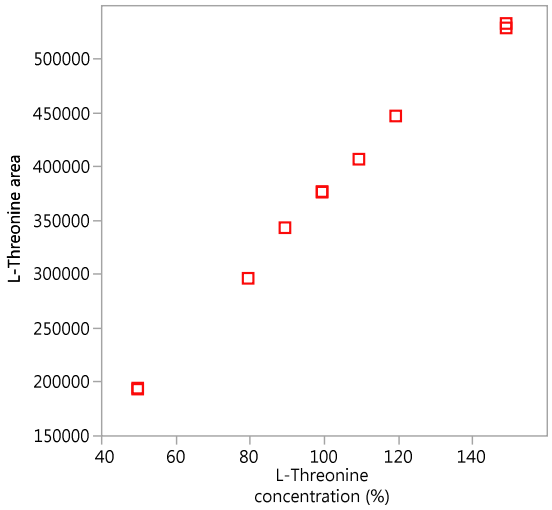
These results show that the equality of the slopes of the regression lines is not rejected, p=0.072>0.05; therefore, no statistical evidence exists at the 5% level to assert that the matrix has a significant effect.

L-Threonine

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=0.9967 and when it´s contained in a matrix, r=0.9975. 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≥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	-590,127	7201,193	-0,082	0,936	-15662,397	14482,143
Conc. (%)	3720,008	69,327	53,659	0,000	3574,905	3865,112

- When the analyte is contained in a complex matrix,

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	29188,000	5800,638	5,032	0,000	17047,124	41328,876
Conc. (%)	3440,330	56,055	61,374	0,000	3323,006	3557,655

The estimates are:

$$a_1 = -590{,}127, b_1 = 3720{,}008 \quad a_2 = 29188{,}000, b_2 = 3440{,}330$$

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.

When the analyte is isolated in the sample the hypothesis of homoscedasticity was not rejected, p=0.067. In this case it can be said that the estimates found by the method of ordinary least squares are valid. When the analyte is contained in a matrix, the homoscedasticity hypothesis was rejected, p=0,000. Therefore, it is concluded, in this case, that the estimates found by the method of ordinary least squares are not valid.

The following tables present the results of the tests:

- the analyte is the only component of the sample,

C (Observed value)	0,540
C (Critical value)	0,561
p-value (unilateral)	0,067
alfa	0,05

- the analyte is contained in a complex matrix,

C (Observed value)	0,898
C (Critical value)	0,561
p-value (unilateral)	0,000
alfa	0,05

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 contained in a complex matrix, 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	7675,864	4229,467	1,815	0,085	-1176,512	16528,239
Conc. (%)	3683,189	40,915	90,020	0,000	3597,552	3768,826

The estimates, when the analyte is the only component of the sample, remain the same:

$$a_1 = -590{,}127,\ b_1 = 3720{,}008 \quad a_2 = 7675{,}864,\ b_2 = 3683{,}189$$

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	247539111729	1	247539111729	2879,241	0,000
Residual	1633501207	19	85973748		
Total	249172612936	20			

Regression Statistics	
R Square	0,9934
Adjusted R Square	0,9931
Standard Error	9272,20
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 99.34% of the variability in the data.

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

When the analyte is contained in a complex matrix,

ANOVA

	SS	*df*	*MS*	*F*	*p-value*
Regression	106299132158	1	106299132158	8103,561	0,000
Residual	249234077	19	13117583		
Total	106548366235	20			

Regression Statistics	
R Square	0,9977
Adjusted R Square	0,9975
Standard Error	3621,82
Observations	21

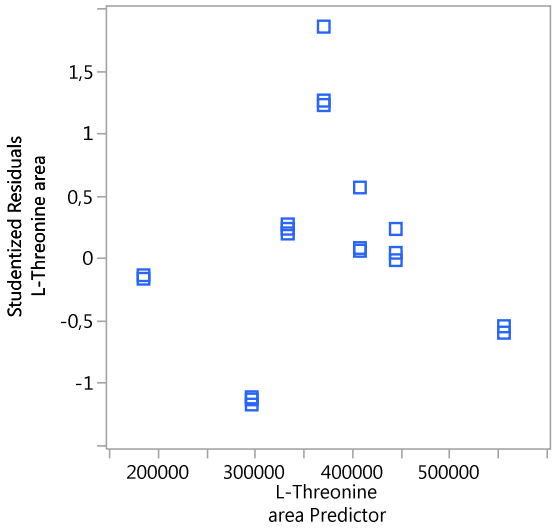
Therefore, the hypothesis of slope being zero is rejected p=0.000<0.05, and the line represents 99.77% of the variability in the data.

Residuals

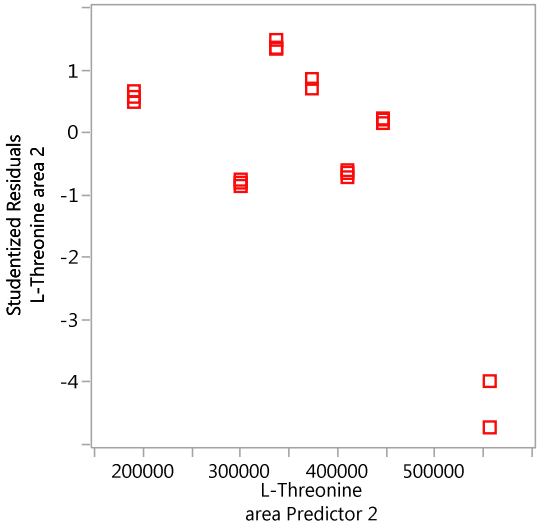
Residuals are extremely useful to check whether a particular linear regression model is appropriate for the data. So a scatterplot graph must be built in which residuals and predictive values for Area are crossed. For the regression line to be appropriate is necessary that the scatterplot does not produce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

$$b_1 = 3720{,}008 \qquad b_2 = 3683{,}189$$

The following table indicates the test results,

Paralell Fit SSE	*Full SSE*	*NDF*	*DDF*	*F Ratio*	*Prob > F*
1890017015,20	1882629844,80	1	38	0,149	0,702

These results show that the equality of the slopes of the regression lines is not rejected, p=0.702>0.05; therefore, no statistical evidence exists at the 5% level to assert that the matrix has a significant effect.

L-Hystidine

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.

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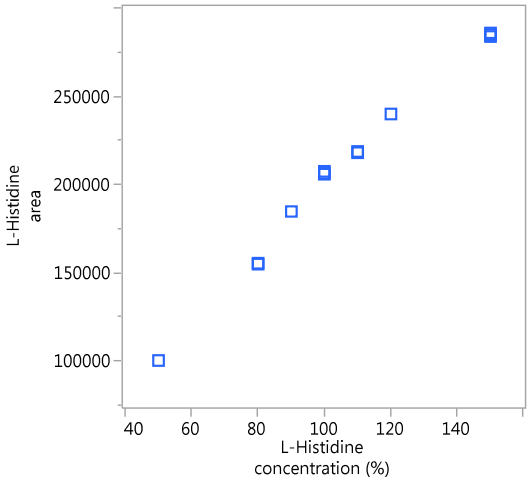
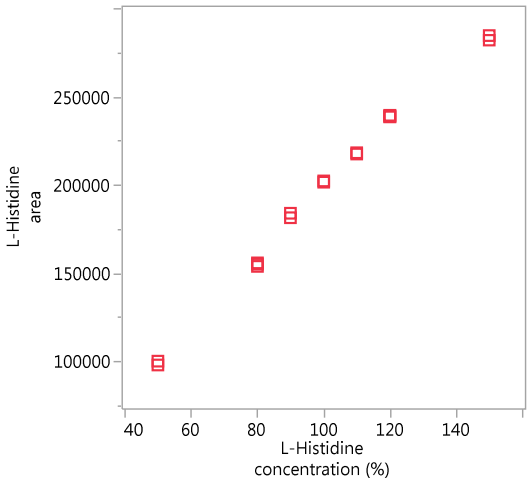


It is also observed an almost perfect linear relationship between Area and Concentration(%) when the analyte is contained in a complex matrix.



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, 0.9900, which was verified. When the analyte is the only component of the sample, r=0.9949; and when the analyte is contained in a complex matrix, r=0.9959.

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	10543,238	4540,115	2,320	0,032	1040,773	20045,911
Conc. (%)	1875,974	43,501	43,120	0,000	1784,927	1967,026

- in the second case,

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	10524,722	4057,307	2,590	0,018	2032,681	19016,764
Conc. (%)	1869,255	38,978	47,960	0,000	1787,674	1950,836

Therefore, we have:

$$a_1 = 10543{,}238 \;\; b_1 = 1875{,}974 \qquad a_2 = 10524{,}722,\; b_2 = 1869{,}255$$

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.

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=0.159 and p=0.734.

The following tables present the results of the tests:

- the analyte is the only component of the sample,

C (Observed value)	0,468
C (Critical value)	0,561
p-value (unilateral)	0,159
alfa	0,05

- the analyte is contained in a complex matrix,

C (Observed value)	0,313
C (Critical value)	0,561
p-value (unilateral)	0,734
alfa	0,05

Therefore, it is concluded that the estimates found by the method of ordinary least squares are valid.

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 ordinary least squares method,

ANOVA

	SS	*df*	*MS*	*F*	*p-value*
Regression	63553648436	1	63553648436	1859,7	0,000
Residual	649299590	19	34173663		
Total	64202948026	20			

Regression Statistics	
R Square	0,9899
Adjusted R Square	0,9894
Standard Error	5845,82
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 98.99% of the variability in the data.

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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	62768355983	1	62768355983	2299,9	0,000
Residual	518544833	19	27291833		
Total	63286900817	20			

Regression Statistics	
R Square	0,9918
Adjusted R Square	0,9914
Standard Error	5224,16
Observations	21

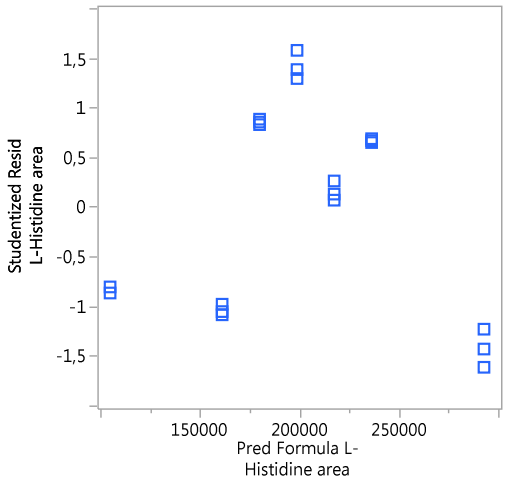
This leads to the conclusion that the hypothesis of slope being zero is rejected p=0.000<0.05, and on the other hand, the line may explain 99.18% of the variability in the data.

Residuals

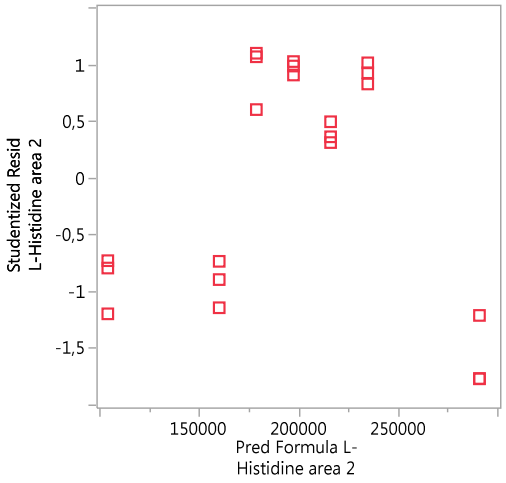
Residuals are extremely useful to check whether a particular linear regression model is appropriate for the data. So a scatterplot graph must be built in which residuals and predictive values for Area are crossed. For the regression line to be appropriate is necessary that the scatterplot does not produce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

$$b_1 = 1875{,}974 \quad \text{and} \quad b_2 = 1869{,}255$$

The result obtained by the test application is,

Paralell Fit SSE	*Full SSE*	*NDF*	*DDF*	*F Ratio*	*Prob > F*
1168251237,00	1167844422,80	1	38	0,013	0,909

Thus, from the Parallelism F test, it was concluded that the equality of the slopes of the regression lines is not rejected, p=0.909>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.

L-Tyrosine

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.

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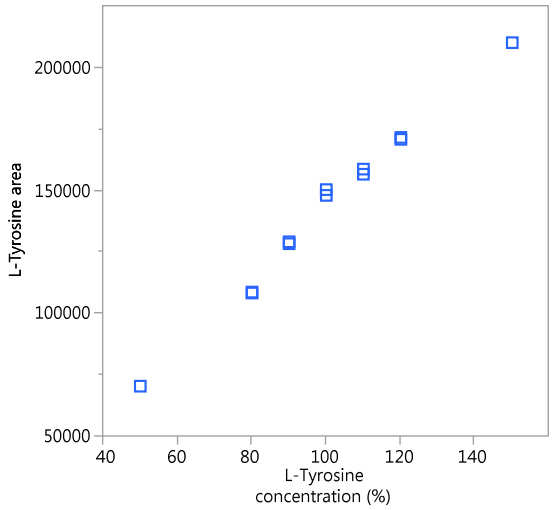
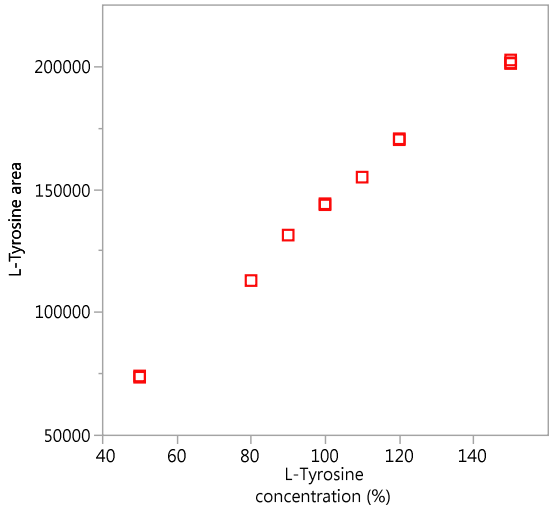
1st case scenario: the scatterplot reveals an almost perfect linear relationship,



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=0.9965 and when it´s contained in a matrix, r=0.9971. 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:

- the analyte is the only component of the sample:

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-158,349	2856,951	-0,055	0,956	-6138,016	5821,318
Conc. (%)	1417,897	27,355	51,834	0,000	1360,643	1475,152

- the analyte is contained in a complex matrix:

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	11519,683	2387,116	4,826	0,000	6523,391	16515,974
Conc. (%)	1299,288	22,932	56,657	0,000	1251,290	1347,286

In short,

$$a_1 = -158{,}349,\ b_1 = 1417{,}897 \quad a_2 = 11519{,}683,\ b_2 = 1299{,}288$$

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.

<u>*Cochran´s C test*</u>
Cochran´s C test must be applied to assess whether the homogeneity assumption is not violated. As in previous analytes this test was applied when:

- the analyte is the only component of the sample,

C (Observed value)	0,525
C (Critical value)	0,561
p-value (unilateral)	0,081
alfa	0,05

- the analyte is contained in a matrix,

C (Observed value)	0,785
C (Critical value)	0,561
p-value (unilateral)	0,001
alfa	0,05

As Cochran´s C test does not reject the hypothesis of homogeneity of variances when the analyte is the only component of the sample, p=0.081>0.05; but reject when the analyte is contained in a complex matrix, p=0.001<0.05.
It is concluded that the estimates found by the method of ordinary least squares are valid only for the first case. For the second case, coefficients must be estimated again, but now with WLS method.

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 in a complex matrix, 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	7764,491	2748,664	2,825	0,011	2011,472	13517,510
Conc. (%)	1358,258	29,372	46,243	0,000	1296,782	1419,734

The estimates, when the analyte is the only component of the sample, remain the same:

$$a_1 = -158{,}349,\ b_1 = 1417{,}897 \quad a_2 = 7764{,}491,\ b_2 = 1358{,}258$$

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 **OLS method**

	SS	*df*	*MS*	*F*	*p-value*
Regression	36356626768	1	36356626768	2686,712	0,000
Residual	257108290	19	13532015		
Total	36613735058	20			

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ANOVA	WLS method				
	SS	***df***	***MS***	***F***	***p-value***
Regression	9178947838	1	9178947838	2138,453	0,000
Residual	81554300	19	4292332		
Total	9260502138	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 0.9930 for the case where the analyte is isolated, meaning that 99.30% of data variability is explained by the line.
R^2 value is 0.9912 considering the other case, meaning that 99.12% of data variability is explained by the line:

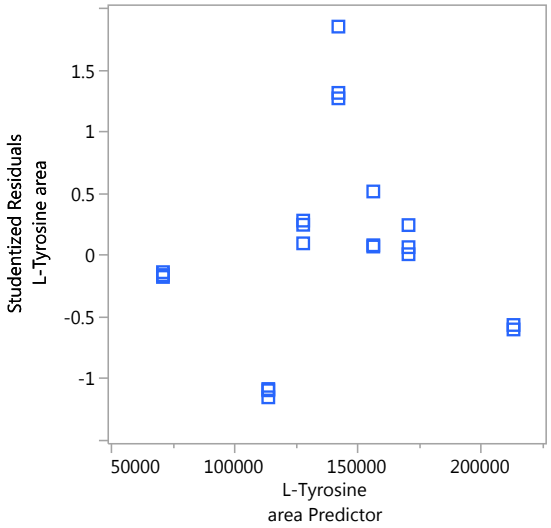
Regression Statistics	
R Square	0,9930
Adjusted R Square	0,9926
Standard Error	3678,59
Observations	21

Regression Statistics	
R Square	0,9912
Adjusted R Square	0,9907
Standard Error	2071,79
Observations	21

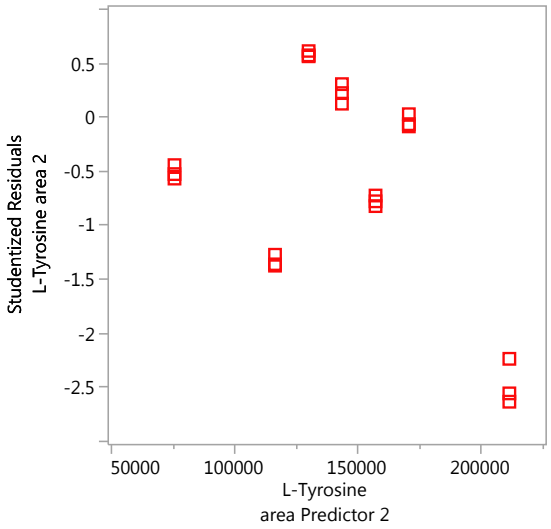
Residuals
Residuals allow you to check the quality of the fit of a linear regression model. So a scatter graph must be built in which residuals and predictive values for Area are crossed. The regression line is well adjusted when the graph does not produce a marked pattern.

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:

$$b_1 = 1417{,}897 \qquad b_2 = 1358{,}258$$

The results of the Parallelism F Test are:

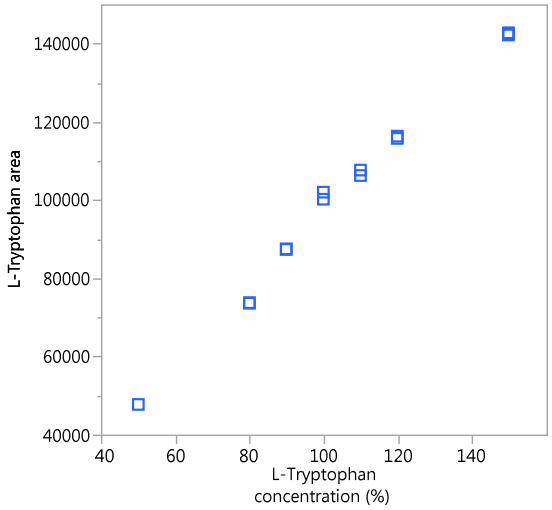
Paralell Fit SSE	*Full SSE*	*NDF*	*DDF*	*F Ratio*	*Prob > F*
352523208,89	338651246,96	1	38	1,557	0,220

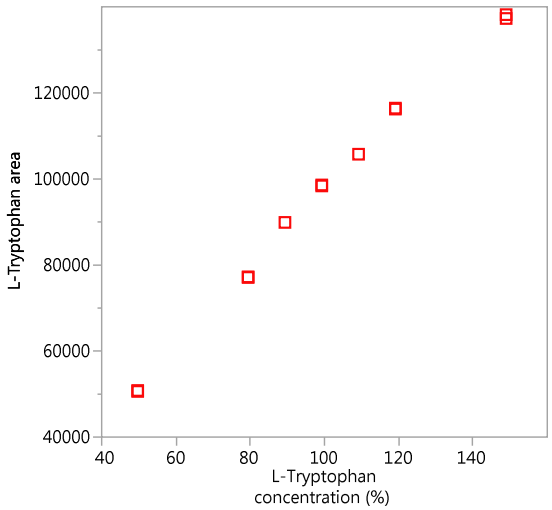
At the 5% significance level, it can be concluded by the Parallelism F test, that the equality of the regression lines slopes is not rejected, p=0.220>0.05; which means that there is no statistical evidence to say that the matrix has a significant effect.

L-Tryptophan

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=0.9966
Analyte is contained in a matrix: r=0.9969

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	300,857	1907,762	0,158	0,876	-3692,135	4293,850
Conc. (%)	965,503	18,364	52,575	0,000	927,067	1003,940

- the analyte is contained in a complex matrix,

	Coefficients	*Std. Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	8476,944	1668,551	5,080	0,000	4984,628	11969,261
Conc. (%)	887,369	16,132	55,008	0,000	853,605	921,133

Where the following estimates were extracted:

$$a_1 = 300{,}857,\ b_1 = 965{,}503 \qquad a_2 = 8476{,}944,\ b_2 = 887{,}369$$

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)	0,539
C (Critical value)	0,561
p-value (unilateral)	0,067
alfa	0,05

- the analyte is contained in a complex matrix,

C (Observed value)	0,833
C (Critical value)	0,561
p-value (unilateral)	0,000
alfa	0,05

As Cochran´s C test does not reject the hypothesis of homogeneity of variances when the analyte is the only component of the sample, p=0.067>0.05; but reject when the analyte is contained in a complex matrix, p=0.000<0.05. It is concluded that the estimates found by the method of ordinary least squares are valid only for the first case. For the second case, coefficients must be estimated again, but now with WLS method.

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 in a complex matrix, 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	5424,208	1641,218	3,305	0,004	1989,099	8859,316
Conc. (%)	930,453	17,661	52,685	0,000	893,489	967,418

The estimates, when the analyte is the only component of the sample, remain the same:

$$a_1 = 300{,}857,\ b_1 = 965{,}503 \qquad a_2 = 5424{,}208,\ b_2 = 930{,}453$$

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	**OLS method**				
	SS	***df***	***MS***	***F***	***p-value***
Regression	16679008705	1	16679008705	2764,17	0,000
Residual	114646044	19	6034002		
Total	16793654749	20			

ANOVA	**WLS method**				
	SS	***df***	***MS***	***F***	***p-value***
Regression	5957766750	1	5957766750	2775,679	0,000
Residual	40781941	19	2146418		
Total	5998548690	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 0.9932

Regression Statistics	
R Square	0,9932
Adjusted R Square	0,9928
Standard Error	2456,42
Observations	21

In the case where it is contained in a matrix is 0.9932

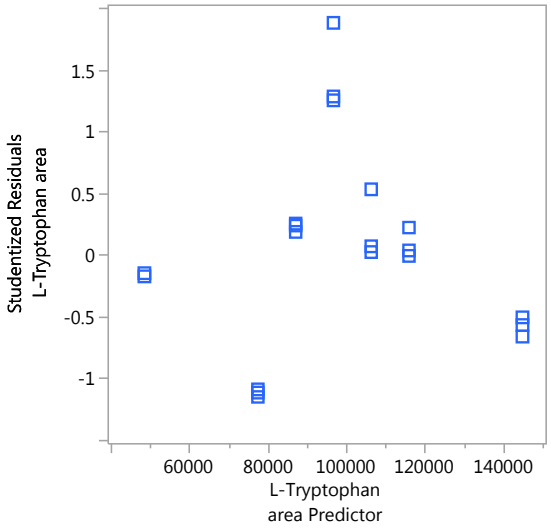
Regression Statistics	
R Square	0,9932
Adjusted R Square	0,9928
Standard Error	1465,07
Observations	21

This means that, in the first case, 99.32% of the variability of the data is explained by the line; while in the second case the percentage is 99.32%, which is an exceptional value.

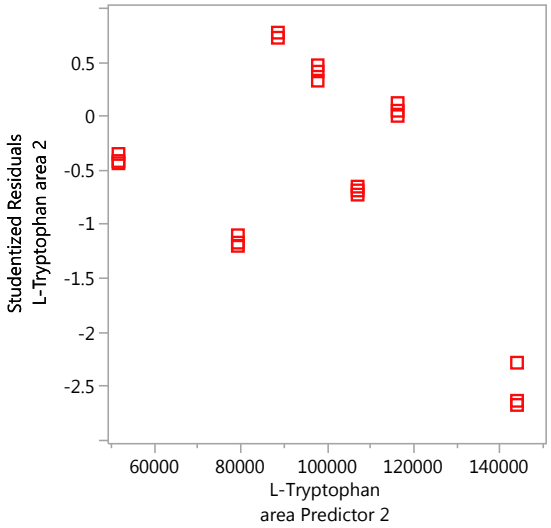
Residuals

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:

$b_1 = 965{,}503$ $b_2 = 930{,}453$

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

Paralell Fit SSE	*Full SSE*	*NDF*	*DDF*	*F Ratio*	*Prob > F*
161531778,21	155423052,21	1	38	1,494	0,229

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=0.229>0.05; 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 measurements of one analyst should not be higher than 3.7%
- RSD between the results of the two analyst should not be higher than 3.7%
- The average of two analytes should not be statistically different (*t Test*)

- L-Lysine acetate

Sample	Analyst 1: SMP Date: 12 Sep. 2016 Assay (%)	Analyst 2: JP Date: 09 Sep. 2016 Assay (%)
A1	96.3	96.0
A2	95.7	94.4
A3	96.0	96.7
A4	96.6	97.9
A5	95.7	96.9
A6	95.1	98.4
Average (%)	**95.9**	**96.7**
RSD (%)	**0.5**	**1.5**

Mean between analysts: 96.3 %
RSD between analysts: 1.2 %

t Test
|t Stat| = -1.276024
t Critical two-tail = 2.570582
t Stat≤t Critical two-tail, the average between the two analysts are not statistically different.

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

- L-Threonine

Sample	Analyst 1: SMP Date: 12 Sep. 2016 Assay (%)	Analyst 2: JP Date: 09 Sep. 2016 Assay (%)
A1	100.5	101.3
A2	99.9	99.2
A3	100.4	101.8
A4	100.9	103.1
A5	100.1	101.8
A6	99.2	104.1
Average (%)	**100.2**	**101.9**
RSD (%)	**0.6**	**1.6**

Mean between analysts: 101.0 %
RSD between analysts: 1.5 %

t Test
|t Stat| = -2.270574
t Critical two-tail = 2.570582
t Stat≤t Critical two-tail, the average between the two analysts are not statistically different.

- L-Histidine

Sample	Analyst 1: SMP Date: 12 Sep. 2016 Assay (%)	Analyst 2: JP Date: 09 Sep. 2016 Assay (%)
A1	101.6	101.4
A2	101.1	98.8
A3	101.3	101.6
A4	102.2	103.0
A5	101.3	101.7
A6	100.2	104.8
Average (%)	**101.3**	**101.9**
RSD (%)	**0.7**	**1.9**

Mean between analysts: 101.6 %

RSD between analysts: 1.4 %

t Test
|t Stat| = -0.654394
t Critical two-tail = 2.570582
t Stat≤t Critical two-tail, the average between the two analysts are not statistically different.

- L-Tyrosine

Sample	Analyst 1: SMP Date: 12 Sep. 2016 Assay (%)	Analyst 2: JP Date: 09 Sep. 2016 Assay (%)
A1	101.2	101.8
A2	100.5	98.8
A3	101.0	102.0
A4	101.6	103.2
A5	100.8	101.7
A6	99.9	104.4
Average (%)	**100.8**	**102.0**
RSD (%)	**0.6**	**1.8**

Mean between analysts: 101.4 %
RSD between analysts: 1.4 %

t Test
|t Stat| = -1.417509
t Critical two-tail = 2.570582
t Stat≤t Critical two-tail, the average between the two analysts are not statistically different.

- L-Tryptophan

Sample	Analyst 1: SMP Date: 12 Sep. 2016 Assay (%)	Analyst 2: JP Date: 09 Sep. 2016 Assay (%)
A1	99.8	101.4
A2	99.4	98.3
A3	99.7	101.4
A4	100.8	102.5

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Sample	Analyst 1: SMP Date: 12 Sep. 2016 Assay (%)	Analyst 2: JP Date: 09 Sep. 2016 Assay (%)
A5	99.6	100.9
A6	98.8	104.0
Average (%)	**99.7**	**101.4**
RSD (%)	**0.7**	**1.8**

Mean between analysts: 100.6 %
RSD between analysts: 1.6 %

t Test
|t Stat| = -2.109573
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%

- L-Lysine acetate

Sample	Assay (%)
A1	96.3
A2	95.7
A3	96.0
A4	96.6
A5	95.7
A6	95.1
Average (%)	**95.9**
RSD (%)	**0.5**

- L-Threonine

Sample	Assay (%)
A1	100.5
A2	99.9
A3	100.4
A4	100.9

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Sample	Assay (%)
A5	100.1
A6	99.2
Average (%)	**100.2**
RSD (%)	**0.6**

- L-Histidine

Sample	Assay (%)
A1	101.6
A2	101.1
A3	101.3
A4	102.2
A5	101.3
A6	100.2
Average (%)	**101.3**
RSD (%)	**0.7**

- L-Tyrosine

Sample	Assay (%)
A1	101.2
A2	100.5
A3	101.0
A4	101.6
A5	100.8
A6	99.9
Average (%)	**100.8**
RSD (%)	**0.6**

- L-Tryptophan

Sample	Assay (%)
A1	99.8
A2	99.4
A3	99.7

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Sample	Assay (%)
A4	100.8
A5	99.6
A6	98.8
Average (%)	**99.7**
RSD (%)	**0.7**

3.3.4.3. Instrumental Repeatability

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

Injection	L-Lysine acetate Area	L-Threonine Area	L-Histidine Area
1	763549	361877	192693
2	765302	362105	189921
3	764886	361286	187231
4	763886	361033	184936
5	762549	360724	189203
6	763809	360890	189069
7	762690	360935	190933
8	762876	360700	188201
9	761655	360390	187243
10	761977	360834	185902
Average	**763318**	**361077**	**188533**
RSD (%)	**0.2**	**0.2**	**1.2**

Injection	L-Tyrosine Area	L-Tryptophan Area
1	140317	96570
2	140512	96583
3	140428	96567
4	140228	96470
5	140156	96391
6	140389	96580
7	140237	96479
8	140007	96405

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Injection	L-Tyrosine Area	L-Tryptophan Area
9	140049	96363
10	139982	96344
Average	**140230**	**96475**
RSD (%)	**0.2**	**0.1**

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%

- L-Lysine acetate

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
80	85.760	87.177	101.653
	85.760	87.898	102.493
	86.015	88.975	103.411
	Mean (%)		102.529
	RSD (%)		0.872
100	107.200	108.764	101.459
	107.200	106.153	99.023
	106.945	104.874	98.064
	Mean (%)		99.515
	RSD (%)		1.759
120	128.640	128.723	100.065
	128.895	130.012	100.866
	128.640	131.218	102.004
	Mean (%)		100.978
	RSD (%)		0.965

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

- L-Threonine

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
80	80.257	81.727	101.831
	79.318	81.461	102.702
	80.257	82.590	102.907
	Mean (%)		102.480
	RSD (%)		0.557
100	99.500	101.907	102.419
	99.969	100.636	100.667
	99.500	99.475	99.974
	Mean (%)		101.020
	RSD (%)		1.247
120	119.682	119.317	99.695
	119.212	120.560	101.131
	118.743	121.754	102.536
	Mean (%)		101.121
	RSD (%)		1.404

- L-Histidine

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
80	80.183	81.661	101.844
	80.183	81.296	101.388
	80.183	81.471	101.606
	Mean (%)		101.613
	RSD (%)		0.225
100	99.243	102.018	102.796
	99.900	101.963	102.066
	100.557	100.051	99.496
	Mean (%)		101.453
	RSD (%)		1.708
120	118.960	120.734	101.492
	119.617	122.192	102.152

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
	119.617	122.501	102.411
	Mean (%)		102.018
	RSD (%)		0.465

- L-Tyrosine

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
80	79.920	80.890	101.213
	80.753	82.625	102.318
	80.753	81.722	101.262
	Mean (%)		101.598
	RSD (%)		0.615
100	99.900	101.224	101.325
	99.900	99.015	99.114
	100.733	98.398	97.683
	Mean (%)		99.374
	RSD (%)		1.846
120	120.713	118.455	98.130
	119.048	120.476	101.200
	120.713	122.175	101.212
	Mean (%)		100.181
	RSD (%)		1.773

- L-Tryptophan

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
80	81.277	83.849	103.164
	80.193	82.498	102.874
	79.110	80.052	101.191
	Mean (%)		102.410
	RSD (%)		1.041
100	100.784	102.059	101.266

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Level (%)	Theoretical concentration (%)	Experimental concentration (%)	Recovery (%)
	99.700	98.573	98.870
	100.784	100.433	99.652
	Mean (%)		99.929
	RSD (%)		1.223
120	118.123	115.798	98.032
	119.207	119.239	100.028
	118.123	120.547	102.052
	Mean (%)		100.037
	RSD (%)		2.010

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**

- L-Lysine acetate

Time point	Area
0 hour	681650
12 hours	690651
24 hours	694196
Average (0–24 hours)	**687923**
RSD (0–24 hours)	**1.3%**

- L-Threonine

Time point	Area
0 hour	356592
12 hours	360635
24 hours	361900
Average (0–24 hours)	**359246**
RSD (0–24 hours)	**1.0%**

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

- L-Histidine

Time point	Area
0 hour	192852
12 hours	193174
24 hours	194972
Average (0–24 hours)	**193912**
RSD (0–24 hours)	**0.8%**

- L-Tyrosine

Time point	Area
0 hour	136054
12 hours	137312
24 hours	138088
Average (0–24 hours)	**137071**
RSD (0–24 hours)	**1.0%**

- L-Tryptophan

Time point	Area
0 hour	93920
12 hours	94911
24 hours	95317
Average (0–24 hours)	**94619**
RSD (0–24 hours)	**1.0%**

The sample solution is stable during 24 hours after preparation.

→ **Standard solution**

- L-Lysine acetate

Time point	Area
0 hour	804077
12 hours	815964
24 hours	820028

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Time point	Area
Average (0–24 hours)	812053
RSD (0–24 hours)	1.4%

- L-Threonine

Time point	Area
0 hour	374904
12 hours	380972
24 hours	381808
Average (0–24 hours)	**379228**
RSD (0–24 hours)	**1.0%**

- L-Histidine

Time point	Area
0 hour	204535
12 hours	207455
24 hours	207181
Average (0–24 hours)	**205858**
RSD (0–24 hours)	**0.9%**

- L-Tyrosine

Time point	Area
0 hour	142788
12 hours	144362
24 hours	145689
Average (0–24 hours)	**144239**
RSD (0–24 hours)	**1.4%**

- L-Tryptophan

Time point	Area
0 hour	97819
12 hours	99421

Time point	Area
24 hours	98809
Average (0–24 hours)	**98314**
RSD (0–24 hours)	**0.7%**

The sample solution is stable during 24 hours after preparation.

3.3.6.2. Variation of column temperature

The column temperature was changed in the range of 50ºC to 60ºC (55ºC ± 5ºC), determining their influence on the retention times, number of theoretical plates (N) and resolution when compared with the original method conditions.

Variation of column temperature	55ºC (method conditions)			50ºC			60ºC		
	rt (min)	N	Resol.	rt (min)	N	Resol.	rt (min)	N	Resol.
L-Lysine	7.350	1770587	10.945	7.349	1829936	12.050	7.303	1754821	10.200
L-Threonine	5.889	401234	48.405	5.955	587312	51.622	5.837	418494	49.492
L-Histidine	3.406	9712	28.409	3.875	25842	32.398	3.449	11568	28.944
L-Tyrosine	7.615	1441871	56.777	7.626	1732143	58.127	7.549	1447252	58.257
L-Tryptophan	8.659	10196398	56.777	8.663	8825815	58.127	8.644	8568785	58.257
Comment	-------			Not critical: slight variation of retention times and theoretical plates. Slight increase and resolution.			Not critical: slight variation of retention times, theoretical plates and resolution.		

3.3.6.3. Variation of column batch

The column batch was changed, determining their influence on the retention times, number of theoretical plates (N) and resolution when compared with the original method conditions.

Variation of column batch	Column internal nº 0175/16			Column internal nº 0163/16		
	rt (min)	N	Resol.	rt (min)	N	Resol.
L-Lysine acetate	7.350	1770587	10.945	7.264	1242470	8.730
L-Threonine	5.889	401234	48.405	5.724	444840	50.360
L-Histidine	3.406	9712	28.409	3.044	12364	37.473
L-Tyrosine	7.615	1441871	56.777	7.499	1219476	50.312

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Variation of column batch	Column internal nº 0175/16			Column internal nº 0163/16		
	rt (min)	N	Resol.	rt (min)	N	Resol.
L-Tryptophan	8.659	10196398	56.777	8.636	3880515	50.312
Comment	-------			Not critical: slight variation of retention times, theoretical plates and resolution.		

3.3.6.4. Variation of the flow rate

The flow rate was changed in the range of 0.6 mL/min to 0.8 mL/min (0.7 mL/min ± 0.1 mL/min), determining their influence on the retention times, number of theoretical plates (N) and resolution when compared with the original method conditions.

Variation of the flow rate	0.7 mL/min (method conditions)			0.6 mL/min			0.8 mL/min		
	rt (min)	N	Resol.	rt (min)	N	Resol.	rt (min)	N	Resol.
L-Lysine acetate	7.350	1770587	10.945	7.528	1752852	11.857	7.178	1772184	10.325
L-Threonine	5.889	401234	48.405	6.213	571141	46.212	5.634	453643	54.744
L-Histidine	3.406	9712	28.409	4.003	14373	27.326	3.324	10714	28.702
L-Tyrosine	7.615	1441871	56.777	7.813	1666066	53.953	7.418	1551530	62.089
L-Tryptophan	8.659	10196398	56.777	8.814	8566856	53.953	8.538	8750615	62.089
Comment	-------			Not critical: slight increase of retention times. Slight variation of theoretical plates and resolution.			Not critical: slight decrease of retention times. Slight variation of theoretical plates and resolution.		

3.3.6.5. Variation of water proportion in mobile phase A

The portion of water in mobile phase A was changed in ± 2% (950 mL ± 19 mL) adjusting also the amount of AccQ-Tag Ultra Eluent A (in order to obtain a total of 1000 mL of mobile phase A), determining their influence on the retention times, number of theoretical plates (N) and resolution when compared with the original method conditions

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by Ultra Performance Liquid Chromatography (UPLC)

Variation of water proportion in mobile phase A	950 mL water + 50mL AccQ-Tag Ultra Eluent A (method conditions)			931 mL water + 69mL AccQ-Tag Ultra Eluent A			969 mL water + 31mL AccQ-Tag Ultra Eluent A		
	rt (min)	N	Resol.	rt (min)	N	Resol.	rt (min)	N	Resol.
L-Lysine acetate	7.350	1770587	10.945	7.329	1868144	10.604	7.312	1750739	12.131
L-Threonine	5.889	401234	48.405	5.848	486386	52.783	5.936	523584	48.878
L-Histidine	3.406	9712	28.409	3.326	12364	32.947	3.825	23303	31.245
L-Tyrosine	7.615	1441871	56.777	7.575	1601769	59.748	7.595	1665414	59.173
L-Tryptophan	8.659	10196398	56.777	8.659	8834416	59.748	8.654	9045898	59.173
Comment	-------			Not critical: slight variation of retention times, theoretical plates and resolution.			Not critical: slight variation of retention times and theoretical plates. Slight increase of resolution.		

3.4. Results analysis

3.4.1. Amino acids assay

3.4.1.1. L-Lysine acetate

The method linearity was demonstrated using the analyte as a single sample component and in the sample matrix in the range of 53.6 to 160.8%. The statistical analysis demonstrates the regression lines equivalency, proving that the sample matrix do not interfere in the L-Lysine quantification.
The mean recovery percentage obtained in the accuracy test is 102.5%, 99.5% and 101.0% 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 96.3% and 1.2%. The mean results are not statistically different.

3.4.1.2. L-Threonine

The method linearity was demonstrated using the analyte as a single sample component and in the sample matrix in the range of 49.8 to 149.5%. The statistical analysis demonstrates the regression lines equivalency, proving that the sample matrix do not interfere in the L-Threonine quantification.
The mean recovery percentage obtained in the accuracy test is 102.5%, 101.0% and 101.1% 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 101.0% and 1.5%. The mean results are not statistically different.

3.4.1.3. L-Histidine

The method linearity was demonstrated using the analyte as a single sample component and in the sample matrix in the range of 50.1 to 150.2%. The statistical analysis demonstrates the regression lines equivalency, proving that the sample matrix do not interfere in the L-Histidine quantification.
The mean recovery percentage obtained in the accuracy test is 101.6%, 101.5% and 102.0% 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 101.6% and 1.4%. The mean results are not statistically different.

3.4.1.4. L-Tyrosine

The method linearity was demonstrated using the analyte as a single sample component and in the sample matrix in the range of 50.1 to 150.4%. The statistical analysis demonstrates the regression lines equivalency, proving that the sample matrix do not interfere in the L-Tyrosine quantification.
The mean recovery percentage obtained in the accuracy test is 101.6%, 99.4% and 100.2% 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 101.4% and 1.4%. The mean results are not statistically different.

3.4.1.5. L-Tryptophan

The method linearity was demonstrated using the analyte as a single sample component and in the sample matrix in the range of 49.9 to 149.6%. The statistical analysis demonstrates the regression lines equivalency, proving that the sample matrix do not interfere in the L-Tryptophan quantification.
The mean recovery percentage obtained in the accuracy test is 102.4%, 99.9% 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 100.6% and 1.6%. The mean results are not statistically different.

3.4.2. Robustness

The sample and standard solutions are stable during 24 hours after their preparation.
The variations tested in the column temperature, column batch, flow rate and water proportion in mobile phase A composition 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 Amino Acids in Ketosteril Drug-Coated Tablets.