14-HYDROXYLATED BRASSINOSTEROID

Collaborative Study

Full Scale Collaborative Study for the Determination of
14-hydroxylated brassinosteroid, by Reversed Phase HPLC

Report to CIPAC
By
Chinese Pesticide Analytical Committee（CHIPAC）

Method Developed by CHENGDU NEWSUN CROP SCIENCE CO., LTD

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1. Participants

By mid of May 2022, 17 of the 18 laboratories provided their results on the determination of 14-Hydroxylated brassinosteroid according to CIPAC Information Sheet No. 331.

One participant (Laboratory 18) could not complete the test in time due to the failure receipt of samples caused by COVID-19 pandemic.

The results for the 17 participants are presented in the following section.

Participating laboratories are listed in randomized sequence as follows, and lab numbers in all tables were assigned according to the chronological order of enrollment.

Kaiwei Shi	Institute for the Control of Agrochemicals, Ministry of Agricultrue and Rural Affairs Maizidian street 22, Chaoyang District, Beijing, P. R. China
Florentina Ciotea	Head of laboratory for quality control of pesticides National Phytosanitary Authority 11 Voluntari Bvld.,VOLUNTARI, Romania
Chunqing Hou, Haixia Wang	Shenyang SYRICI Testing Co., Ltd. China Shenyang Chemical Research Institute, No.8 shenliao East Road, Tiexi District, Shenyang city, China
Cornel Grecu	Alchimex quality control laboratory ALCHIMEX SA, 63-ALEXANDRU CONSTANTINESCU – 011472, BUCHAREST-1, ROMANIA.
Hongxia Li	Nutrichem Laboratory Co., Ltd. No. 27, Life Science Park Road, Changping Dist., Beijing 102206, P. R. China
Angela Santilio	National Institute of Health (Istituto Superiore di Sanità) Department of Environmental and Health ECASS Section - Pesticide Unit V.le Regina Elena 299 00161 ROME (ITALY)
Elen Karasali，Petros Tsiantas	Benaki Phytopathological Institute 8 Stefanou Delta Street, Kifissia, Athens, 14561 Greece.
Jim Garvey	Department of Agriculture, Food and The Marine,Ireland The Food Chemistry Laboratories, Backweston Laboratory Complex, Backweston, Celbridge W23 X3PH, Co. Kildare, Ireland
Agus Salim	Laboratorium PT Agriculture Construction - Indonesia Jl. Siliwangi No 68，Bogor，Jawa Barat，Indonesia
Aiping Xu	Laprode (Zhejiang) analysis Co., Ltd 4/F, Building 6, No.503 Xingguo Road, Yuhang District, Hangzhou, Zhejiang P.R. China
Judy Dong	BNS GLP TEST FACILITY 199 Fenghuang 8th Road, Bincheng District, Binzhou City, Shandong Province, China
Wendy Wang	Jiangsu Agrochem Laboratory Co., Ltd No.98, Minjiang Road, Hi-Tech Development Zone Changzhou, Jiangsu, China
Bo Zhang	Central Research Institute of China Chemical Science and Technology Co., Ltd 309, Beijiao building, No. 20 Xueyuan Road, Haidian District, Beijing, China

Lily Yan	Jiangsu Rotam Chemistry Co., Ltd No.88 Rotam Road, ETDZ, Kunshan, Jiangsu, 215301, China
Jianzhong Yu	Institute of Agro-product Safety and Nutrition, Zhejiang Academy of Agricultural Sciences 1-5040, New Area of Zhejiang Academy of Agricultural Sciences, No. 198, Shiqiao Road, Hangzhou, Zhejiang, China
Zhiyu He	Guizhou Jiandee Technology Co., LTD Baijin road No.3491,Baiyun district, Guiyang, P. R. China
Lu Huang	Hunan Research Institute of Chemical Industry Testing Technology Co., Ltd. No. 550, Changsha Avenue, Lituo street, Yuhua District, Changsha City, Hunan Province，China
Javier García-Hierro Navas	Laboratorio Arbitral Agroalimentario, Aguarón, 13. E28023 Madrid, Spain

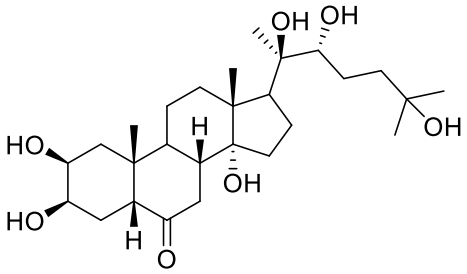
2. 14-Hydroxylated brassinosteroid, General Information

Chemical name: (2β,3β,5β,22R)-2,3,14,20,22,25-hexahydroxy-Cholestan-6-one

Common name: 14-Hydroxylated brassinosteroid

CAS-Number: 457603-63-3

Structure:



Molecular mass: 482.7 g/mol

Empirical formula: $C_{27}H_{46}O_7$

3. Samples

In February 2022, Information Sheet No. 331 was sent out by the CIPAC Secretary inviting members to participate in a collaborative study on the determination of 14-hydroxylated brassinosteroid by reversed phase HPLC

Five test samples (described below), including the 14-Hydroxylated brassinosteroid analytical reference standard and derivatization reagent were shipped to the participants:

A) 14-Hydroxylated brassinosteroid TK-1
B) 14-Hydroxylated brassinosteroid TK-2
C) 14-Hydroxylated brassinosteroid SL-1
D) 14-Hydroxylated brassinosteroid SL-2
E) 14-Hydroxylated brassinosteroid SL-3

14-Hydroxylated brassinosteroid analytical reference standard (99.8% purity)

All participants except for Laboratory 18 (total of 17 laboratories) sent back their results in time.

4. Method

4.1 Scope

The content of 14-hydroxylated brassinosteroid in technical concentrate and in formulated products (soluble liquid) was determined.

4.2 Principle

The content of 14-hydroxylated brassinosteroid in technical concentrate and the formulation (SL) is determined by HPLC on Eclipse Plus 100 x 4.6 mm (i.d) columns, C18 packed with octadecyl silane filler (3.5 µm), or equivalent, with mobile phase composed of acetonitrile/water (45/55 v/v) and UV detection at 222 nm after phenylboronic acid derivatization. Quantitation is done by external standardization.

4.3 Procedure

Samples should be analyzed in duplicate at two different days resulting in a total of four individual test results for each sample. All test solutions should be prepared freshly on Day 2.

5. Remarks of the Participants

Participants made comments about the performance of the method and noted deviations from the method. Below is a summary of specific method conditions provided by the participating laboratories.

Lab Number	HPLC-System	Mobile phase:	Flow rate:	Column	wavelength	Injection volume:	Column Temp:	Remarks
Laboratory 1	Shimadzu-20 AT	Acetonitrile : aquabidest = 45 : 55 (v/v)	1.0 mL/min	Thermohypersyl C-18 (150 x 4.6 mm id, 5 µm)	222 nm	10 µL	30 °C	
Laboratory 2	Agilent 1200	TK: Acetonitrile:Water=45:55(v/v) SL: time Acetonitrile: Water(v/v) 00.00 45 55 15.00 45 55 15.01 85 15 20.00 85 15 20.01 45 55 25.00 45 55	0.8 mL/min	InfinityLab Poroshell 120 EC-C18 (2.7 Micron with Column ID, 100mm*4.6mm)	222 nm	10 µL	30 °C	
Laboratory 3	Agilent 1260 Infinity Ⅱ	TK: Acetonitrile : Water= 45 : 55 (v/v); SL: Time [min], Acetonitrile[% v/v] : Water[% v/v] 0, 45 : 55; 11, 45 : 55; 12, 85 : 15; 16, 85 : 15; 17, 45 : 55; 19, 45 : 55;	1.0 mL/min	Agilent ZORBAX Eclipse Plus C18 (3.5 µm, octadecyl silane filler, 100 mm, 4.6 mm)	222 nm	10 µL	30 °C	

Laboratory 4	Agilent, DAD, 1260 series	consistent with CIPAC method completely	1.0 mL/min	Agilent Eclipse Plus (100mm*4.6mm （i.d.), C18 3.5μm)	222 nm	10 μL	30 °C	
Laboratory 5	SHIMADZU LC-20A	TK: Acetonitrile / Water= 45 / 55 (v/v)	1.0 mL/min	Eclipse Plus C18 (4.6 mm × 150 mm, 5 μm)	222 nm	10 μL	30 °C	Mobile phase for SL was modified due to the change of column length, the conditions were altered as follows: Acetonitrile / Water= 0-15 min, 45 / 55 (v/v); 15-16 min, 85 / 15 (v/v); 16-19 min, 85 / 15 (v/v); 19-20 min, 45 / 55 (v/v); 20-28 min, 45 / 55 (v/v).
Laboratory 6	SHIMADZU LC20A	（TK） Acetonitrile/Water=43/57（v/v）（ 20min） （SL） Acetonitrile/Water=43/57（0-15min） 95/5（15.1-19min） 43/57（19.1-27min）	0.8 mL/min	Stainless stell Eclipse Plus C18 (100mm×4.6mm（i.d） 3.5um)	222 nm	10 μL	35 °C	
Laboratory 7	Shimadzu LC-30A	TK Sample: Acetonitrile / Water= 45 / 55 (v/v)	1.0 mL/min	Agilent eclipse plus C18 (100 mm, 4.6 mm, 3.5 μm)	222 nm	10 μL	30 °C	Mobile phase for SL Sample: 0~11 min, Acetonitrile / Water= 45 / 55 (v/v) 12~16 min, Acetonitrile / Water= 85 / 15 (v/v) 17~19 min, Acetonitrile / Water= 45 / 55 (v/v) Retention time:

								approximately 8.9 min
Laboratory 8	Agilent 1260	Acetonitrile / Water=45 / 55 (v/v)	1.0 mL/min	Eclipse XDB-C18 (5μm，C18, 150mm*4.6mm)	222 nm	10 uL	30 °C	
Laboratory 9	Agilent Technologies 1260 Infinity II	TK：Acetonitrile/Water=45/55(v/v) SL：Acetonitrile/Water 45/55(v/v)，0-11 min； 85/15(v/v)，12-16 min； 45/55(v/v)，17-22 min;	1.0 mL/min	ZORBAX Eclipse Plus (C18 packed with octadecyl silane filler (3.5 μm), 100 mm×4.6 mm(i.d))	222 nm	10 μL	30 °C	
Laboratory 10	Agilent 1260-DAD	Acetonitrile / Water (45:55)	1.0 mL/min	Phenomemex Kinetex® (2.6 μm, C18 110 Å, 100 mm x 4.6 mm)	222 nm	10 μL	30 °C	
Laboratory 11	Agilent 1100	Acetonitrile/Water=45/55(v/v)	1.0 mL/min	ZORBAX SB-C18 (150 mm×4.6 mm(i.d.), 5 μm)	222 nm	10 μL	30 °C	
Laboratory 12	Thermo Vanquish	Water 55 % Acetonitrile 45 %	1.0 mL/min	Phenomenex Kinetex C18 (100x4.6 mm,2.6 μm)	222 nm	10 μL	30 °C	Bad asymmetry peak. Injection volume is high, column must be cleaned carefully.
Laboratory 13	Agilent Technoloies 1260 infinity	TK: Acetonitrile/Water=45+55 (V/V) SL: 0 min, Acetonitrile/Water=45+55 (V/V); 12 min, 85+15 (V/V)； 16 min, 85+15（V/V）; 17 min, 45+55（V/V）; 22 min, 45+55（V/V）;	1.0 mL/min	Agilent Poroshell 120 CS-C18 (4.6×100 mm, 2.7 μm)	222 nm	10 μL	30 °C	
Laboratory 14	Thermo UltiMate 3000 RS	Acetonitrile / Water 45/55 (v/v)	0.3 mL/min	Phenomenex Kinetex (2.6um C18 100A, 100 x 3 mm)	222 nm	5 μL	30 °C	

Laboratory 15	1200 System with DAD	(TK) Acetonitrile :Water= 45:55 (v/v) (0.0075%SL) Time Acetonitrile : water(v/v) 0min 45:55 11min 45:55 12min 85:15 16min 85:15 17min 45:55 19min 45:55	1.0 mL/min	ZORBAX Eclipse XDB-C18 (4.6mm*150mm, 5um)	222 nm	10 µL	30 °C	
Laboratory 16	PERKIN ELMER, FLEXAR	Water/Acetonitrile; ISO 55:45 v/v; Gradient: as described in the method	1.0 mL/min	Eclipse Plus C18 (100 x 4.6 mm, 3.5 um)	222 nm	10 uL	30 °C	The sample SL was concentrated to nearly dry with the rotary evaporator at 70 °C. The method should describe this condition.
Laboratory 17	SIMADZU LC-20AB	A: H2O & B: ACN	1.15 mL/min	PARTISIL 5 ODS3 (100 x 4.6 mm,5 µm)	222 nm	10 µL	25 °C	SL sample preparation: Concentration to nearby dry was done with rotary evaporation at 90 °C

6. Evaluation and Discussion

6.1 Evaluation of the Quality of Data and Chromatograms

The data obtained from each of the laboratories were reviewed to determine if there were any significant deviations regarding the chromatography which might affect the analysis results.
Visual examination of the chromatograms showed no evidence for invalid data.
All other changes and observations noted by the 17 participants were not expected to affect the analysis results significantly.

6.2 Determination of 14-Hydroxylated brassinosteroid

Results reported by the laboratories and the statistical evaluation are listed in tables 1-4 and displayed in figures 1-5.

The statistical evaluation of the data was done following the “Guidelines for CIPAC Collaborative Study Procedures for Assessment of Performance of Analytical Methods”, according to DIN ISO 5725. The data were examined for outliers and stragglers using Mandel’s k-statistics on the within-lab variance, followed by Mandel’s h-statistics on the lab means, and iterating where necessary. The tests were performed at an alpha level of 0.01 for outlier (marked with **), and 0.05 for straggler (marked with *).

A comparison of the RSD_R of this collaborative study with the unmodified Horwitz equation showed that the relative reproducibility standard deviation (RSD_R) is above the Horwitz value for all the formulations without elimination of stragglers and outliers (see Table 3). The relative reproducibility standard deviation (RSD_R) is below the Horwitz value for all the samples (TK-1, TK-2, SL-1, SL-2 and SL-3) with elimination of stragglers and outliers and no more than three Lab results have been removed per sample (see Table 4). The Horwitz Ratio (HorRat) was found within the desired range (0.3-1.0). Due to the universal applicability of the method, this collaborative trial is acceptable.

Table 1: 14-Hydroxylated brassinosteroid (g/kg); Results for each laboratory on day 1 and day 2;

	14-Hydroxylated brassinosteroid TK-1		14-Hydroxylated brassinosteroid TK-2		14-Hydroxylated brassinosteroid SL-1		14-Hydroxylated brassinosteroid SL-2		14-Hydroxylated brassinosteroid SL-3	
	Day1	Day2	Day1	Day2	Day1	Day2	Day1	Day2	Day1	Day2
Lab 1	817.69	820.46	809.72	803.32	0.0823	0.0817	0.0771	0.0776	0.0754	0.0765
Lab 2	832.27	840.69	844.52	838.85	0.0899	0.0903	0.0845	0.0850	0.0817	0.0820
Lab 3	833.60	830.72	835.41	833.16	0.0833	0.0840	0.0804	0.0788	0.0779	0.0811
Lab 4	831.80	835.75	832.19	831.03	0.0777	0.0808	0.0730	0.0777	0.0773	0.0835
Lab 5	876.90	876.75	875.56	878.49	0.0810	0.0814	0.0770	0.0777	0.0756	0.0769
Lab 6	801.29	799.33	799.08	799.42	0.0741	0.0744	0.0749	0.0751	0.0743	0.0740
Lab 7	814.06	814.66	831.90	831.05	0.0792	0.0800	0.0739	0.0741	0.0719	0.0721
Lab 8	845.31	845.93	840.40	838.06	0.0835	0.0850	0.0771	0.0765	0.0771	0.0781
Lab 9	838.71	839.91	839.76	845.34	0.0863	0.0872	0.0809	0.0833	0.0787	0.0823
Lab 10	830.28	833.46	834.57	832.06	0.0836	0.0820	0.0723	0.0773	0.0728	0.0721
Lab 11	841.40	837.72	852.96	840.61	0.0848	0.0831	0.0828	0.0822	0.0829	0.0822
Lab 12	853.44	851.86	852.85	852.26	0.0788	0.0758	0.0788**	0.0678**	0.0768**	0.0639**
Lab 13	836.93	839.18	828.41	825.98	0.0756	0.0743	0.0685	0.0684	0.0674	0.0675
Lab 14	840.58	827.17	855.61	836.00	0.0321	0.0330	0.0373	0.0310	0.0319	0.0280
Lab 15	806.93	806.81	809.64	810.35	0.0758	0.0764	0.0765	0.0765	0.0765	0.0760
Lab 16	798.77**	844.43**	821.33**	866.21**	0.0851**	0.0772**	0.0873*	0.0781*	0.0933	0.0969
Lab 17	775.32	773.30	774.80	774.20	0.0786	0.0748	0.0760	0.0749	0.0760	0.0740

* Mandel's k-statistic straggler

** Mandel's k-statistic outlier

Table 2: Mean values

	14-Hydroxylated brassinosteroid TK-1	14-Hydroxylated brassinosteroid TK-2	14-Hydroxylated brassinosteroid SL-1	14-Hydroxylated brassinosteroid SL-2	14-Hydroxylated brassinosteroid SL-3
Lab 1	819.08	806.52	0.0820	0.0774	0.0760
Lab 2	836.48	841.68	0.0901	0.0848	0.0819
Lab 3	832.16	834.29	0.0837	0.0796	0.0795
Lab 4	833.78	831.61	0.0793	0.0754	0.0804
Lab 5	876.83*	877.03*	0.0812	0.0774	0.0763
Lab 6	800.31	799.25	0.0743	0.0750	0.0742
Lab 7	814.36	831.48	0.0796	0.0740	0.0720
Lab 8	845.62	839.23	0.0843	0.0768	0.0776
Lab 9	839.31	842.55	0.0868	0.0821	0.0805
Lab 10	831.87	833.32	0.0828	0.0748	0.0725
Lab 11	839.56	846.78	0.0840	0.0825	0.0826
Lab 12	852.65	852.55	0.0773	0.0733	0.0704
Lab 13	838.05	827.20	0.0750	0.0685	0.0675
Lab 14	833.87	845.81	0.0326**	0.0342**	0.0300**
Lab 15	806.87	809.99	0.0761	0.0765	0.0763
Lab 16	821.60	843.77	0.0812	0.0827	0.0951
Lab 17	774.31**	774.50**	0.0767	0.0755	0.0750

* Mandel's h-statistic straggler
** Mandel's h-statistic outlier

Table3: Summary of the statistical evaluation - no elimination of any outliers /stragglers

	TK-1	TK-2	SL-1	SL-2	SL-3
xm [g/kg]	829.22	831.62	0.0780	0.0747	0.0745
xm [% w/w]	82.92	83.16	0.0078	0.0075	0.0075
L	17	17	17	17	17
S_r	8.42	8.90	0.0018	0.0030	0.0028
S_R	23.43	24.27	0.0125	0.0114	0.0132
S_L	21.86	22.59	0.0124	0.0110	0.0129
r	23.57	24.91	0.0050	0.0084	0.0078
R	65.59	67.97	0.0351	0.0320	0.0369
RSD_r	1.01	1.07	2.29	4.00	3.75
RSD_R	2.83	2.92	16.06	15.28	17.69
RSD_R (Hor)	2.06	2.06	8.30	8.36	8.36
HorRat	1.37	1.42	1.93	1.83	2.12

Table4: Summary of the statistical evaluation with elimination Mandel's h and k Statistic Stragglers /Outliers

	TK-1	TK-2	SL-1	SL-2	SL-3
xm [g/kg]	830.28	831.59	0.0809	0.0771	0.0778
xm [% w/w]	83.03	83.16	0.0081	0.0077	0.0078
L	14	14	15	14	15
S_r	3.38	4.88	0.0012	0.0014	0.0017
S_R	15.02	16.36	0.0046	0.0042	0.0064
S_L	14.64	15.61	0.0045	0.0040	0.0062
r	9.46	13.67	0.0034	0.0040	0.0047
R	42.06	45.80	0.0130	0.0119	0.0179
RSD_r	0.41	0.59	1.52	1.87	2.15
RSD_R	1.81	1.97	5.73	5.49	8.23
RSD_R (Hor)	2.06	2.06	8.26	8.32	8.31
HorRat	0.88	0.96	0.69	0.66	0.99

TK-1: Results of Lab 5, 16, 17 were eliminated;
TK-2: Results of Lab 5, 16, 17 were eliminated;
SL-1: Results of Lab 14, 16 were eliminated;
SL-2: Results of Lab 12, 14, 16 were eliminated;
SL-3: Results of Lab 12, 14 were eliminated;

X_m = overall sample mean
L = number of laboratories
S_r = repeatability standard deviation
RSD_r = relative repeatability standard deviation
r = repeatability limit
S_R = reproducibility standard deviation
RSD_R = relative reproducibility standard deviation
R = reproducibility limit
S_L = "pure" between laboratory standard deviation
RSD_R (Hor) = relative reproducibility standard deviation (Horwitz equation)

Figures 1 – 5 (all results)

Fig. 1:

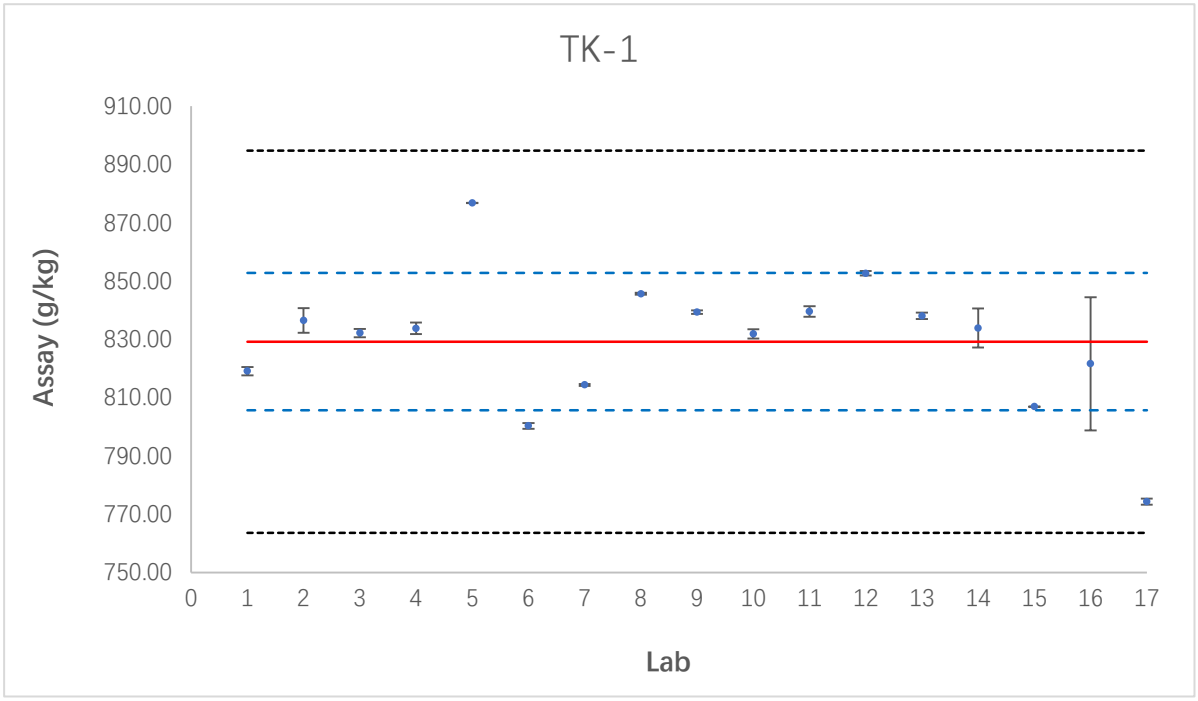


Fig. 2:

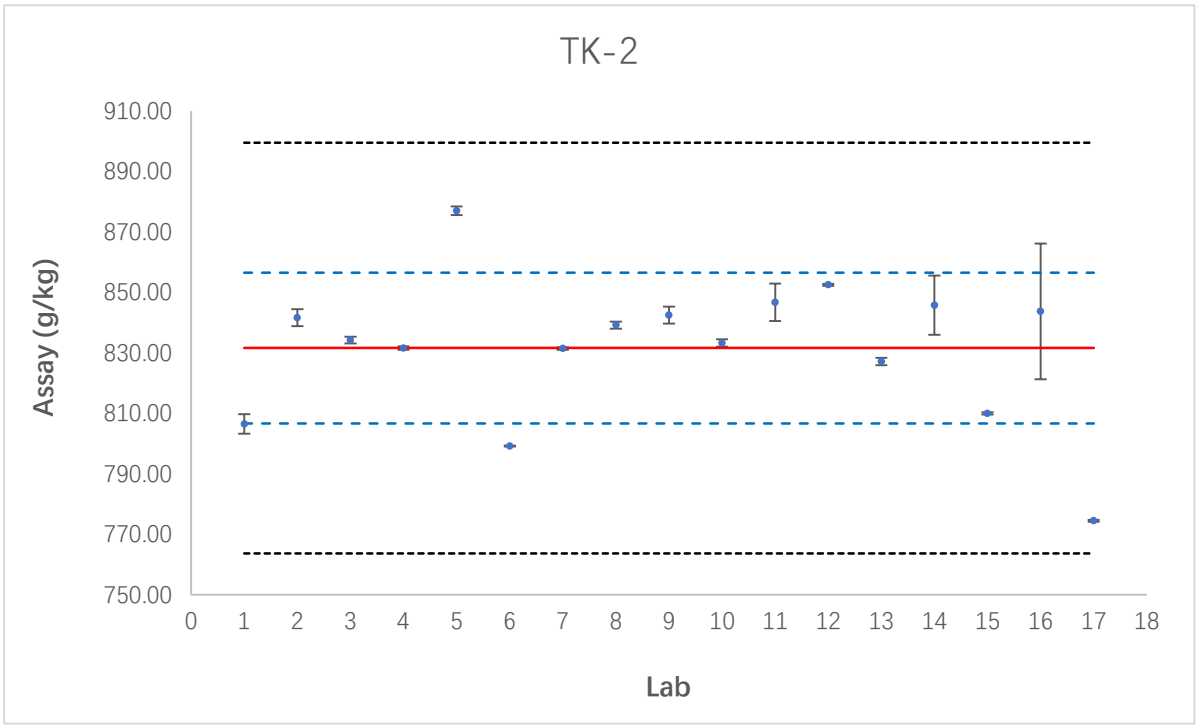


Fig. 3:

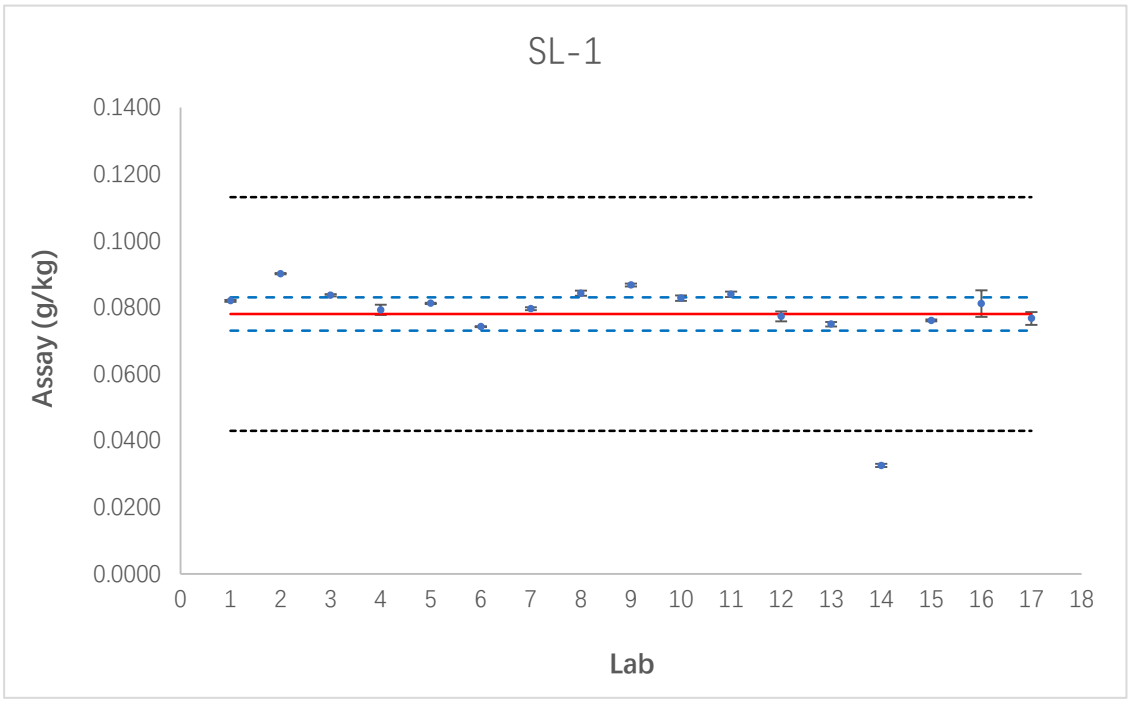

SL-1
Assay (g/kg)
0.1400
0.1200
0.1000
0.0800
0.0600
0.0400
0.0200
0.0000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Lab

Fig. 4:

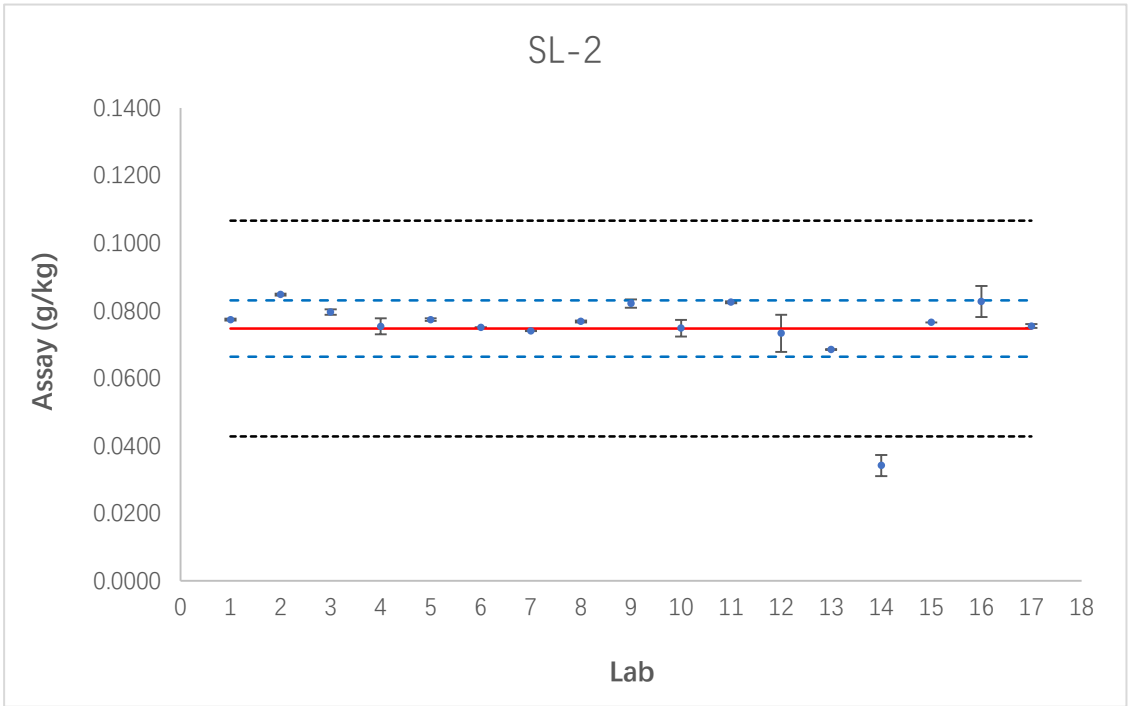
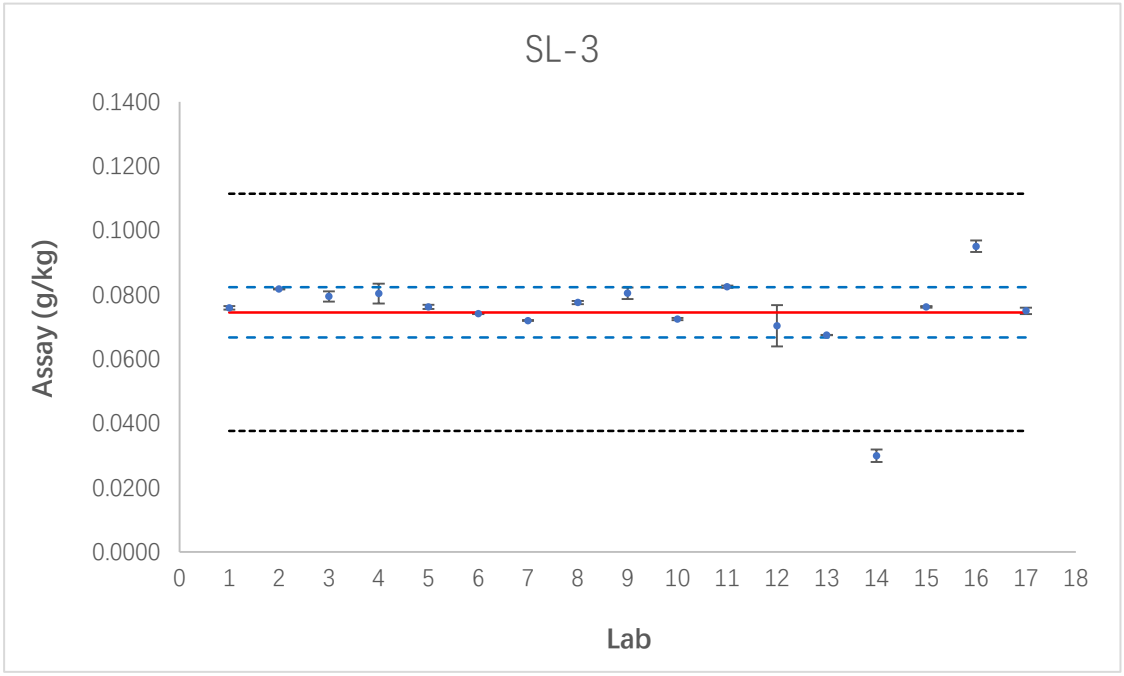

SL-2
Assay (g/kg)
0.1400
0.1200
0.1000
0.0800
0.0600
0.0400
0.0200
0.0000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Lab

Fig. 5:


SL-3
Assay (g/kg)
0.1400
0.1200
0.1000
0.0800
0.0600
0.0400
0.0200
0.0000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Lab

7. Conclusions

17 different laboratories participated in this collaborative study. The results of the labs are given in Table 1-2, the statistical summary is given in Table 4-5. The results are illustrated in figures 1 – 5.

With elimination of outliers and stragglers, the between lab experimental Relative Reproducibility Standard Deviation (% RSD_R) is below the calculated acceptable value based on the Horwitz curve calculation (% RSD_R (Hor)) for all samples. The HorRat values were all within the required range by employing this method. The minimum number of considered results after elimination of stragglers and outliers was 14.

Taking into account the relatively high number of participating laboratories a broad basis was given even after elimination of the outliers. Therefore, CHIPAC considers this method to be suitable and recommend accepting it as a provisional CIPAC method for the determination of 14-Hydroxylated brassinosteroid in both technical concentrate and its associated formulated products.