Indian Journal of Advances in Chemical Science

Article

Simultaneous Determination of Isoquercitrin and Astragalin in Plant (Leaf) Extract Using Liquid Chromatography with Tandem Mass Spectrometry Method for the Application of Toxicology Studies in Matrix

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ABSTRACT

An analytical approach has been developed and validated using liquid chromatography (LC)-mass Spectrometry (MS)/MS for the simultaneous determination of isoquercitrin and astragalin from the TGT Primaage (plant [leaf] extract), following the application of toxicology studies. The purpose of analytical method validation was established a sensitive data, which was investigated for chronic toxicology studies evaluation. The method validations were implemented for determining the individuality and concentration of the analytes, matrix match effects and provide an estimated analytical method validation. The method validation was carried out by performing different parameters using reference standards and test substance solutions of isoquercitrin and astragalin in the matrix and analyzed onto LC-MS/MS. This analytical validated method was successfully applied to the actual samples of the toxicology studies for the dose formulation analysis of TGT Primaage (plant [leaf] extract) samples which had the contents of isoquercitrin and astragalin.

Key words: Flavonoid, Liquid chromatography-Mass spectrometry/mass spectrometry, Toxicology studies, International Conference on Harmonization Q2 (R1), Extraction technique, Matrix effect, Chemical compound.

1. INTRODUCTION

Isoquercitrin is used as a flavonoid and also used as a chemical compound [1]. It is having the 3-O-glucoside of quercetin and it can be isolated from various plant species including *Mangifera indica* (mango) [2]. It is also present in the leaves of *Annona squamosa* and *Camellia sinensis* (tea). *C. sinensis* is a species of green shrub whose leaves and leaf buds are used to harvest tea. It is genus *Camellia* of flowering plants in the Theaceae family. Common names include "tea plant," "tea shrub," and "tea tree" *C. sinensis,* and *C. sinensis assamica* are two major varieties grown today. White tea, yellow tea, green tea, oolong, dark tea, and black tea are harvested from one or the other but are processed differently to attain varying levels of oxidation. Kukicha is also harvested from *C. sinensis* nevertheless uses twigs and stems rather than leaves [3-5]. Isoquercitrin is presently being investigated for the prevention of thromboembolism in selected cancer patients and as an anti-fatigue agent in kidney cancer patients treated with sunitinib [6]. There is a single case report of its use in the successful treatment of prurigo nodularis, a difficult to treat pruritic eruption of the skin [7].

Astragalin is a chemical compound and it can be isolated from *Phytolacca americana* (the American pokeweed) or in the methanolic extract of fronds of the fern *Phegopteris connectilis*. It is also found in wine. Astragalin is also known as 3-O-glucoside of kaempferol [8]. Astragalin is found in alcoholic beverages and especially present in red wine. It is isolated from many plant species such as *P. americana* (the American pokeweed) [9]. Kaempferol 3-O-beta-D-glucoside is a kaempferol O-glucoside in which a glucosyl residue is attached at position three of kaempferol through a beta-glycosidic linkage. It is a kaempferol O-glucoside, a monosaccharide derivative, a trihydroxy flavone, and a beta-D-glucoside. It has a role as a trypanocidal drug and a plant metabolite [10].

The objectives of the present work are (i) to develop and validate the analytical method for the determination of isoquercitrin and astragalin of plant (leaf) extract of TGT Primaage samples in the matrix, (ii) to optimize the extraction method for the determination of analytes at a lower concentration level for the dose formulation analysis of toxicology studies, (iii) to achieve the minimum concentration of test substance for which acceptable recovery will be obtained and reported, and (iv) to perform the analytical method validation for the analysis of the frequent samples.

2. EXPERIMENTAL

2.1. Test Substance

TGT Primaage was provided by The Mitomasa SDN BHD, MALAYSIA, which having the contents of isoquercitrin: 2.06% w/w and astragalin (Kaempferol 3-glucoside): 0.74% w/w. The test substance appearance was dark goldenrod powder.

2.2. Standard Materials

Isoquercitrin (Source: HWI group) and astragalin (Kaempferol 3-glucoside) (Source: Sigma-Aldrich) were purchased.

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ISSN NO: 2320-0898 (p); 2320-0928 (e)
DOI: 10.22607/IJACS.2020.802005

Received: 05th February 2020;
Revised: 08th March 2020;
Accepted: 08th April 2020

2.3. Instrument and Apparatus

Liquid chromatography (LC)-MS/MS (Model: API 4000, Make: AB Sciex) coupled with high-performance liquid chromatograph (HPLC) (Model: Nexera X2, Make: Shimadzu). The Analytical Balance (Model: GR-202, Make: Adair and Dutt), Microbalance (Model: MAY 5/2Y, Make: Radwag), Refrigerator (Model: Enerji, Make: Siemens), Micropipette (Make: Eppendorf), Vortex Shaker (Model: Spinix, Make: Tarsons), and Ultrasonicate cleaning bath (Model: UCB70, Make: SPECTROLAB) were used.

2.4. Solvents and Chemicals

Acetonitrile (Grade: HPLC, Source: J.T. Baker), Water (Grade: Milli-Q water, Source: Merck), Formic Acid (Grade: Emsure, Source: Merck), and Sodium Hydroxide (NaOH) (Grade: Laboratory Reagent, Source: Sigma-Aldrich) were used.

2.5. Methodology

The optimization and validation of the analytical method using the LC separation of mixtures with multiple components and mass spectrometry (MS) provide the structural identity of the individual components with high molecular specificity as well as the detection sensitivity was performed using the LC with tandem MS (LC-MS/MS). The tuning of compounds was performed by precursor and substance ion scanning and compound optimization on a mass spectrometer. The compound retention, better peaks response, and increasing for chromatography optimization efficiency were achieved by selecting the HPLC column. The establishment of sensitivity and consistent ionization of the reproducible response for the target analyte were optimized by selecting an appropriate solvent system for the mobile phase composition.

2.6. Standard Extraction Technique

An aliquot of 100 µL was transferred into 1.0 mL microcentrifuge tube. Thereafter, 100 µL of 100 mM NaOH buffer was added to the same microcentrifuge tube and vortex thoroughly.

A volume 10 µL aliquoted from the processed sample solution and addition of 990 µL of diluent (Acetonitrile: Milli-Q water (80:20), v/v). Mixed well the sample solution and analyzed onto LC-MS/MS.

Note: At the lower concentration level of the dose formulation sample in the matrix 1.0 mg/mL samples, a volume of 100 µL aliquoted from the processed sample solution and addition of 900 µL of diluent and mixed well then analyzed on to LC-MS/MS.

2.7. LC Condition

Different aliquots of standard solutions and sample solution were performed using the analytical method parameter of LC condition. Analytical LC, an instrument fitted with a binary pump, an autosampler injector setup (50 µL loop) with 10°C temperature. The analysis of analytical method validation, a column (Make: Waters X-bridge, C-18 [150×4.6 mm, 3.5 µm particle size]) was suitable with a gradient elution mode mobile phase as described in Table 1 with the flow rate of 1.5 mL/min. The analysis was conducted under column oven temperature 30°C using injection volume 5 µL of desired sample solutions. The analysis run time was 5.0 min and analytes retention times were approximately 1.89 and 2.0 for isoquercitrin and astragalin, respectively.

2.8. MS Condition

Analytes were detected with (Model: API-4000 triple quadrupole mass spectrometer, Make: AB-Sciex) in negative electrospray ionization mode. The isoquercitrin and astragalin concentrations in the matrix were determined with the linear regression by 1/X weighting. The sample acquisition and quantification were performed by Analyst® software version 1.6.2. Mass transitions to produce optimum sensitivity were determined by injecting standards as described in Table 2. The optimized MS variables value were Curtain Gas (CUR) 10 psi, Collision Gas (CAD) 8 psi, Entrance Potential (EP) −5 V, Collision Cell Exit Potential (CXP) −5 V, Dwell Time (Milli Seconds) 200, Ion Spray Voltage −4500 V, and Temperature 500°C, GS1 50 psi, and GS2 60 psi.

Table 1: Gradient time programs for liquid chromatography.

Time (min)	Module	Value	
		Pump A (%) 0.1 % formic acid in Milli-Q water	Pump B (%) acetonitrile
0.0	Pump	80	20
2.0	Pump	40	60
3.0	Pump	40	60
3.1	Pump	80	20
5.0	Pump	80	20

2.9. Calibration Curve, Limit of Detection, and Limit of Quantitation (LOQ)

Calibration curves were generated by serial dilutions of isoquercitrin and astragalin standards mixture. The reference standards working solutions were processed, as per the proposed standard extraction procedure and analytical method parameters. Thereafter, the reference standards were analyzed onto LC-MS/MS. The peak area was plotted against concentrations (µg/L). The correlation coefficient (r), slope (b), and intercept (a) were calculated. The lower LOQ was processed the minimum concentration, which could be detected with a signal-to-noise ratio (S/N) of ≥10:1, was considered as LOQ.

2.10. Precision, Accuracy and Stability of Dose formulation

Precision and accuracy were established at the level of the test concentrations used during the toxicology study (1.0, 100.0, and 300.0 mg/mL) along with the control using test substance. The dose formulation samples were collected from three layers for the homogeneity. The samples were extracted by the proposed extraction technique and analyzed by LC-MS/MS. The analyte concentration in each replicate, the mean of analyte concentration, standard deviation (SD), and % relative SD (RSD) were calculated and reported.

3. RESULTS AND DISCUSSION

The specificity of the method for the determination of isoquercitrin and astragalin concentration in dose formulation was studied by injecting solvent (acetonitrile), diluent, blank matrix (RO Water), reference standards solution, and test item solution. Since, there were no interference between the peaks of the analyte; solvent and diluent for isoquercitrin and astragalin. The method was specific for the analyte. The system suitability solution of reference standard was injected onto LC-MS/MS and observed values of % RSD for area counts of isoquercitrin and astragalin that were NMT 5.0% (Figures 1-12, please see the supplementary information). The linearity for precision, accuracy, intermediate precision, and stability at lower, middle, and higher dose levels in matrix was established by injecting seven different concentrations 2500.620–500,624.600 µg/L of isoquercitrin and 2508.849–500,272.000 µg/L of astragalin working standards solutions and the peak area was plotted against concentration (µg/L). The intercept with Y-axis (a) and slope of the

line (b) were calculated and regression equation was established. The value of correlation coefficient (r>0.99) was obtained. (Figures 13 and 14, please see the supplementary information). The LOQ was determined by injecting different concentrations of isoquercitrin and astragalin solution. The minimum S/N ≥10:1 was considered for LOQ for both the compounds. The lowest quantifiable concentration (LOQ) of isoquercitrin and astragalin in matrix with S/N of 10.1 and 10.6 was calculated, respectively. The precision (% RSD), intermediate precision, accuracy (% recovery), and stability of dose formulation were determined by analyzing dose formulation in matrix (RO water) fortified with test substance at the lower (1.0 mg/mL), middle (100.0 mg/mL), and higher (300.0 mg/mL) dose level as mentioned in Table 3 for isoquercitrin and Table 4 for astragalin. The stock solution stability of reference standard solutions of isoquercitrin and astragalin was performed by comparing with the freshly prepared stock solutions with the prepared and stored reference standard solution in refrigerator condition (2–8°C) of isoquercitrin and astragalin onto LC-MS/MS for 12 days intervals successfully. The robustness experiment was performed by analyzing samples with deliberated changes in proposed analytical method parameters and checked the method capacity. It was concluded that the analytical method found robust.

Initially, the test substance was directly analyzed by dissolved in solvent and diluent against the reference standards solutions for the recovery comparison. Also evaluated the matrix match effects by adding test substance in the matrix (RO water) and % recovery was not observed within the acceptance limits [3,10-12]. The removal of chlorophylls before extraction is essential for the extraction of flavonoids from the test samples [13]. The technique was also available for the procedure of chlorophylls removal with benzene. The trial was not adopted due to benzene comes under International Conference on Harmonization (ICH) (Class-I) chemicals and it is highly carcinogenic [14]. Another approach for the extraction technique was taken by treating the test sample solution with an alkaline solution for the chlorophyll isolation. If heating of green vegetables, processed with an alkaline solution such as NaOH causes the replacement of methyl alcohol on the chlorophyll molecule by sodium (Na^+) ions and the reaction made the chlorophyll water soluble. The test solution was directly mixed with three different concentrations strength of NaOH solutions (i.e., 50 mM, 100 mM, and 200 mM) without heating for reproducible results and samples were further diluted ten folds with diluent. The final samples were filtered through 0.45 µm PVF syringe filter and analyzed onto LC-MS/MS. The % recovery was improved and found within the acceptable limit of 90–110%. The achievement of multiple trials of extraction technique was taken and obtained desired results for 100 mM NaOH solution. The strength value of NaOH solution was chosen for the final analytical method validation.

Table 2: Optimized operational conditions for LC-MS/MS of target analytes.

Parameter	Isoquercitrin		Astragalin (Kaempferol 3-glucoside)	
MRM transitions	463.200/300.100	463.200/271.100	447.400/284.200	447.400/255.100
Declustering potential (DP) V	-135	-110	-110	
Collision energy (CE) eV	-40	-60	-37	-51

Table 3: Evaluation of validation of isoquercitrin results in matrix of test substance TGT Primaage.

Limit of quantification (LOQ) (µg/L)		199.948				
	Fortification level (mg/mL)	Result	Stability of dose formulation			Intermediate precision
			0 h	24 h	48 h	
Precision (% RSD) (Acceptance criteria: ≤10%)	(1.0)	1.27	1.71	0.95	2.55	2.56
	(100.0)	0.68	0.36	1.02	2.69	1.55
	(300.0)	1.13	1.22	1.84	1.07	2.01
Accuracy (% recovery) (Acceptance criteria: 85–115 %)	(1.0)	111.13	98.00	97.04	95.49	96.29
	(100.0)	108.03	97.91	95.68	94.67	92.47
	(300.0)	102.49	99.36	96.40	97.15	93.90

Table 4: Evaluation of validation of astragalin results in matrix of test substance TGT Primaage.

Limit of quantification (LOQ) (µg/L)		71.826				
	Fortification level (mg/mL)	Result	Stability of dose formulation			Intermediate precision
			0 h	24 h	48 h	
Precision (% RSD) (Acceptance criteria: ≤10%)	(1.0)	1.45	0.35	0.76	1.94	3.75
	(100.0)	1.29	1.10	1.57	0.29	3.79
	(300.0)	0.50	1.47	2.15	2.05	2.11
Accuracy (% recovery) (Acceptance criteria: 85–115%)	(1.0)	110.45	100.61	100.56	97.45	100.64
	(100.0)	108.27	99.41	99.40	96.41	99.13
	(300.0)	103.18	100.08	98.72	99.66	98.90

4. CONCLUSION

The results of the method validation fulfilled all the criteria of the Commission Directive "Validation of Analytical Procedures: Text and Methodology" ICH Q 2 (R1) current step 4 version, parent guideline dated October 27, 1994 (Complimentary guideline on methodology dated November 6, 1996, incorporated in November 2005). The analytical method validation is concluded that the method is sensitive, precise, and accurate for the determination of isoquercitrin and astragalin in dose formulation of TGT Primaage in the matrix (RO water) for the toxicology studies.

5. ACKNOWLEDGMENT

This work was supported and conducted by Jai Research Foundation, NGCMA (GLC/C-0031) approved GLP facility for the pre-clinical CRO, India. The authors are grateful to Mr. Kunjan Shah for the leading of the toxicology studies "Repeated Dose 90-Day Oral Toxicity Study of TGT Primaage Through Oral Gavage in Wistar Rats" and Dr. Manish V. Patel for obliging conversation. The Mitomasa SDN BHD, MALAYSIA, has given the opportunities for conducting the experiment work by providing the test substance (TGT Primaage) and approval of the research publication.

6. REFERENCES

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****Bibliographical Sketch***

Mr. Meet Patel, M.Sc. from Valsad (District), Gujarat, India. Post Graduated in (2012) from Sardar Patel University, Vallabh Vidyanagar, Gujarat, India and specialised in Life Sciences (Biotechnology). I have an experience of over 7 years in Medical Device, Pharmaceutical and Agro-chemical Industries (Analytical-R&D) Sector. Currently, I am accompanying with Jai Research Foundation, a pre-clinical Contract Research Organization as Research Officer in Department of Chemistry. Also have an expertise in performing the analytical method validation for pharmaceutical and medical device products following the International Council for Harmonisation for Pharmaceuticals and Agro-chemical products following the SANCO guidelines to meet technical requirements of the regulatory authorities.

SUPPLEMENTARY INFORMATION

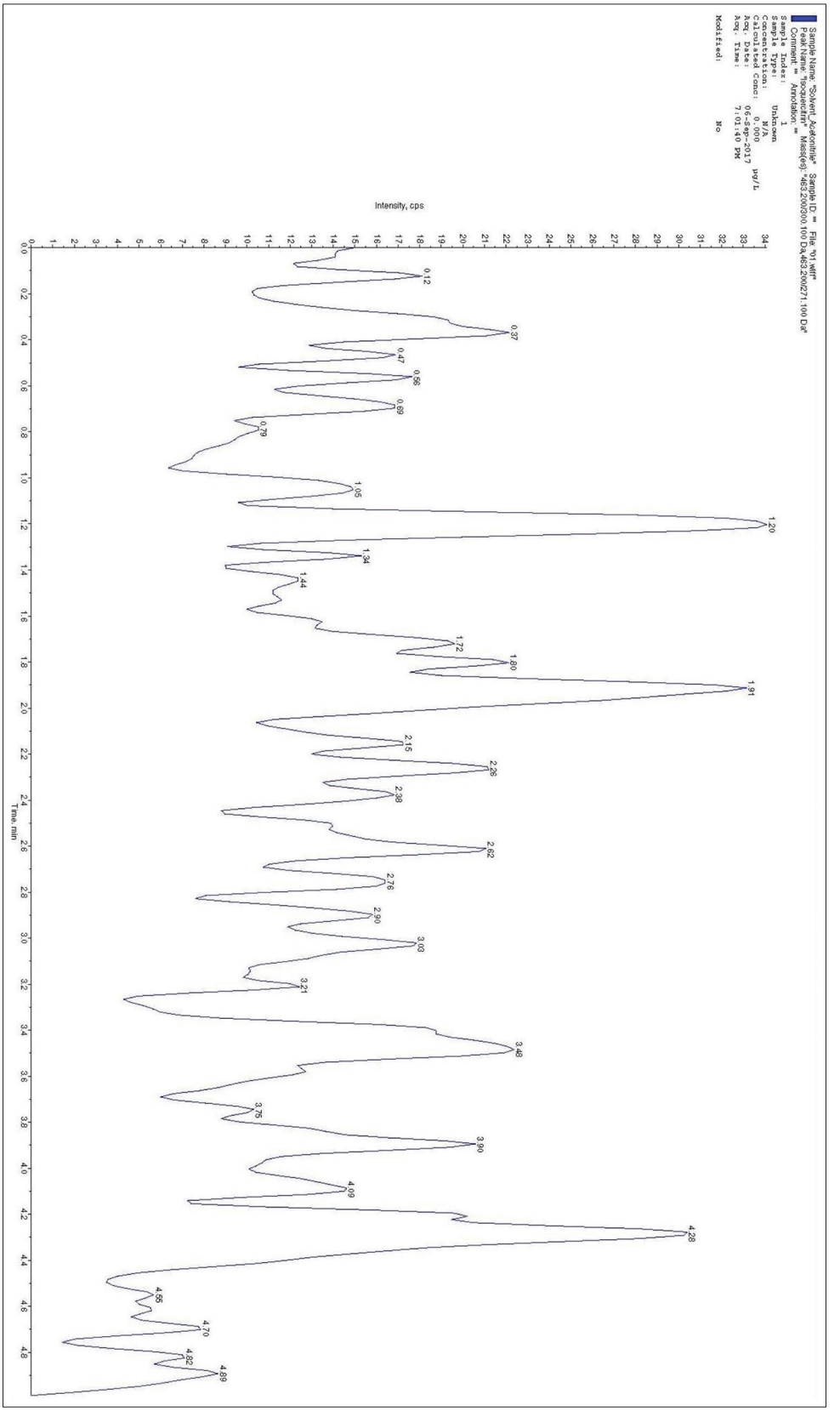

Figure 1: Solvent (Acetonitrile) of Isoquercitrin.

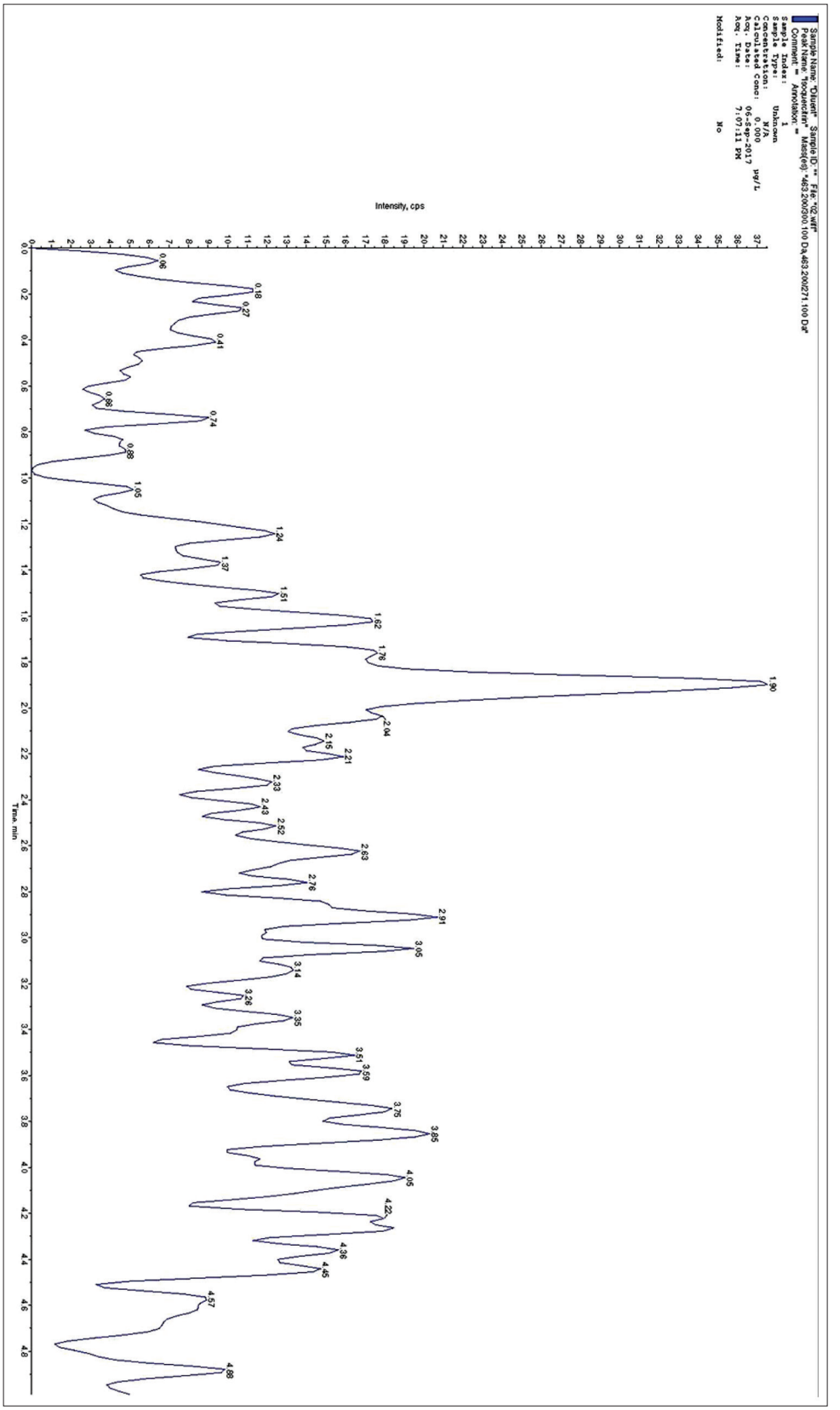

Figure 2: Diluent - (Acetonitrile: Milli-Q water (80:20), v/v) of Isoquercitrin.

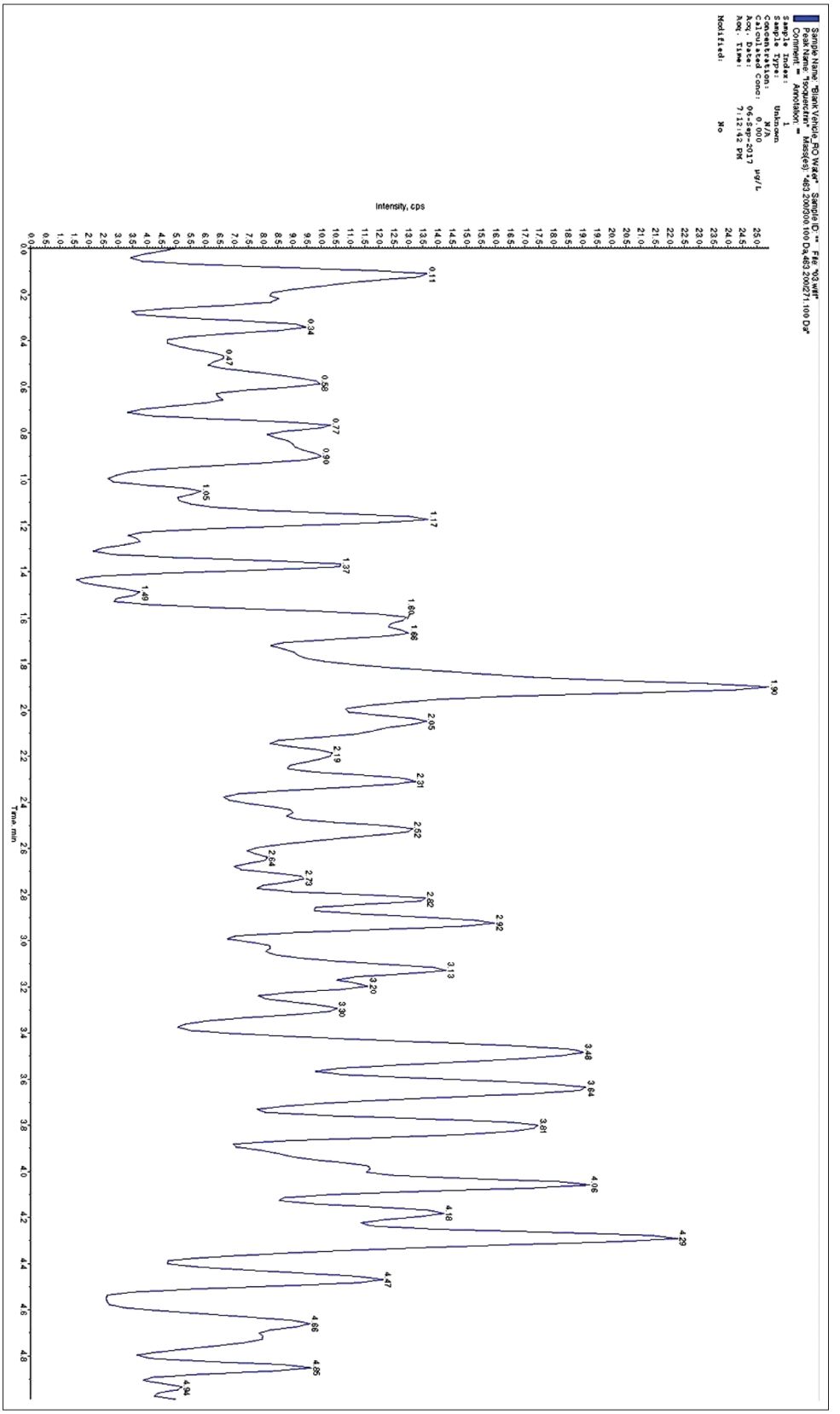

Figure 3: Blank Matrix (RO Water) of Isoquercitrin.

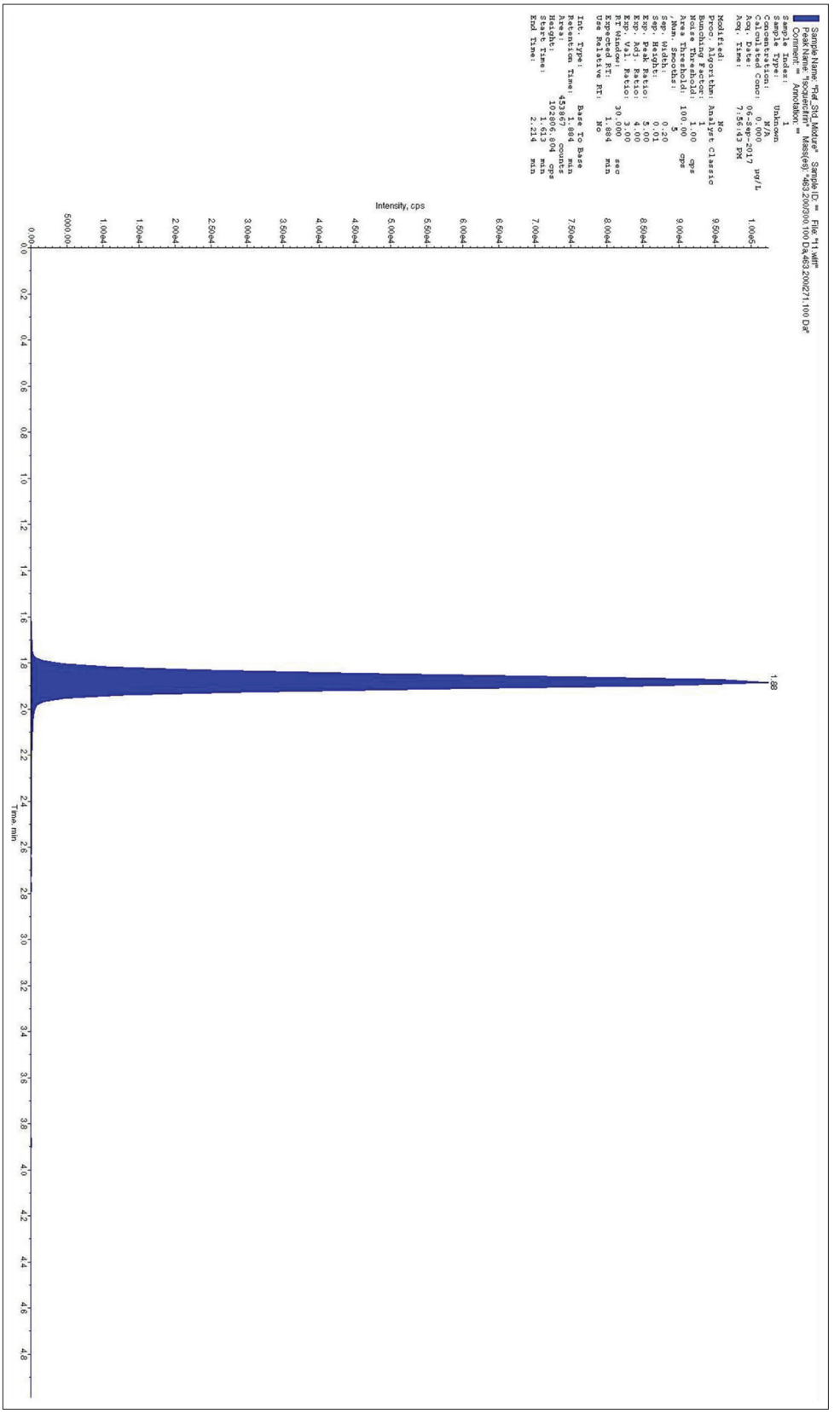

Figure 4: Reference Standard Solution of Isoquercitrin.

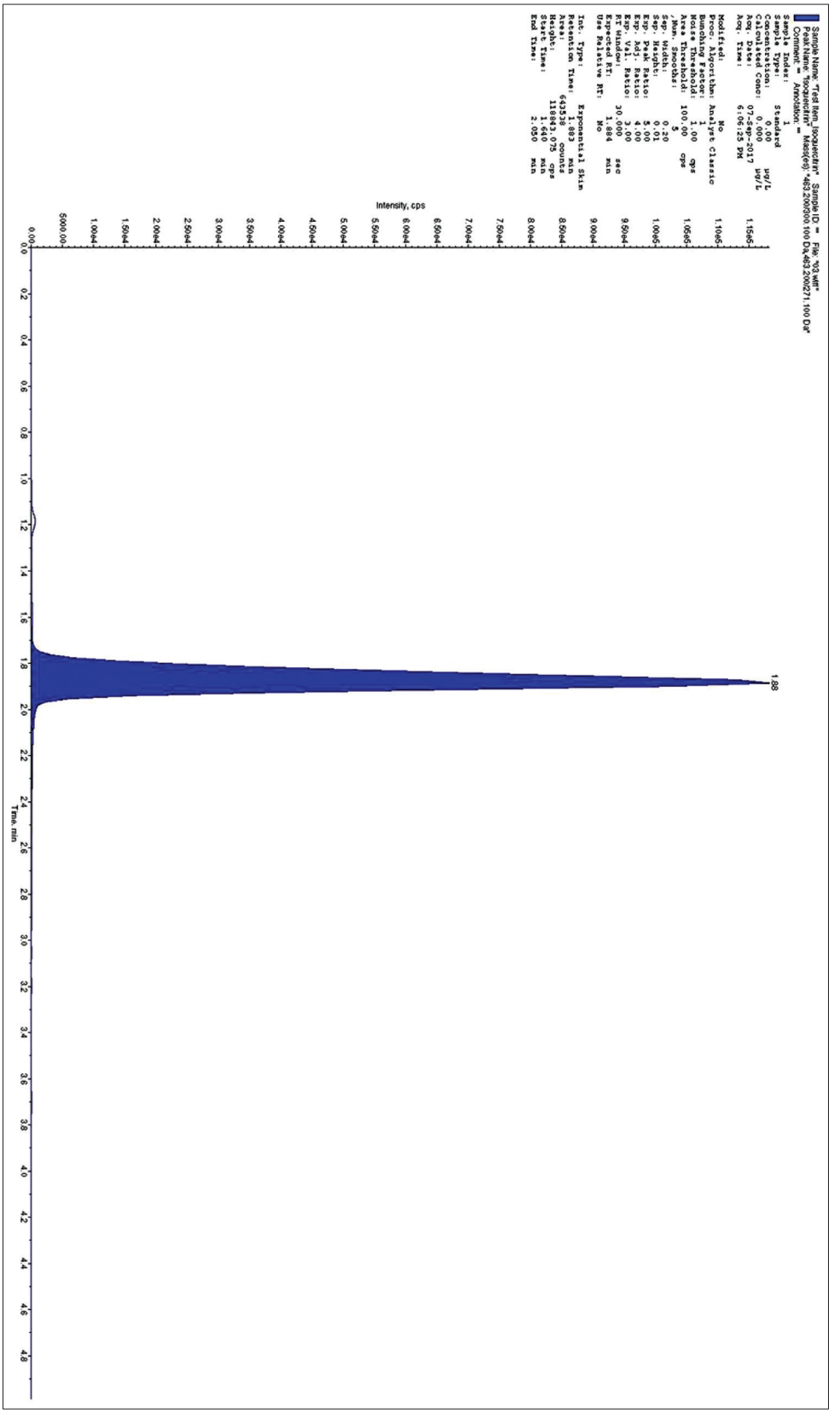

Figure 5: Test Substance Solution of Isoquercitrin.

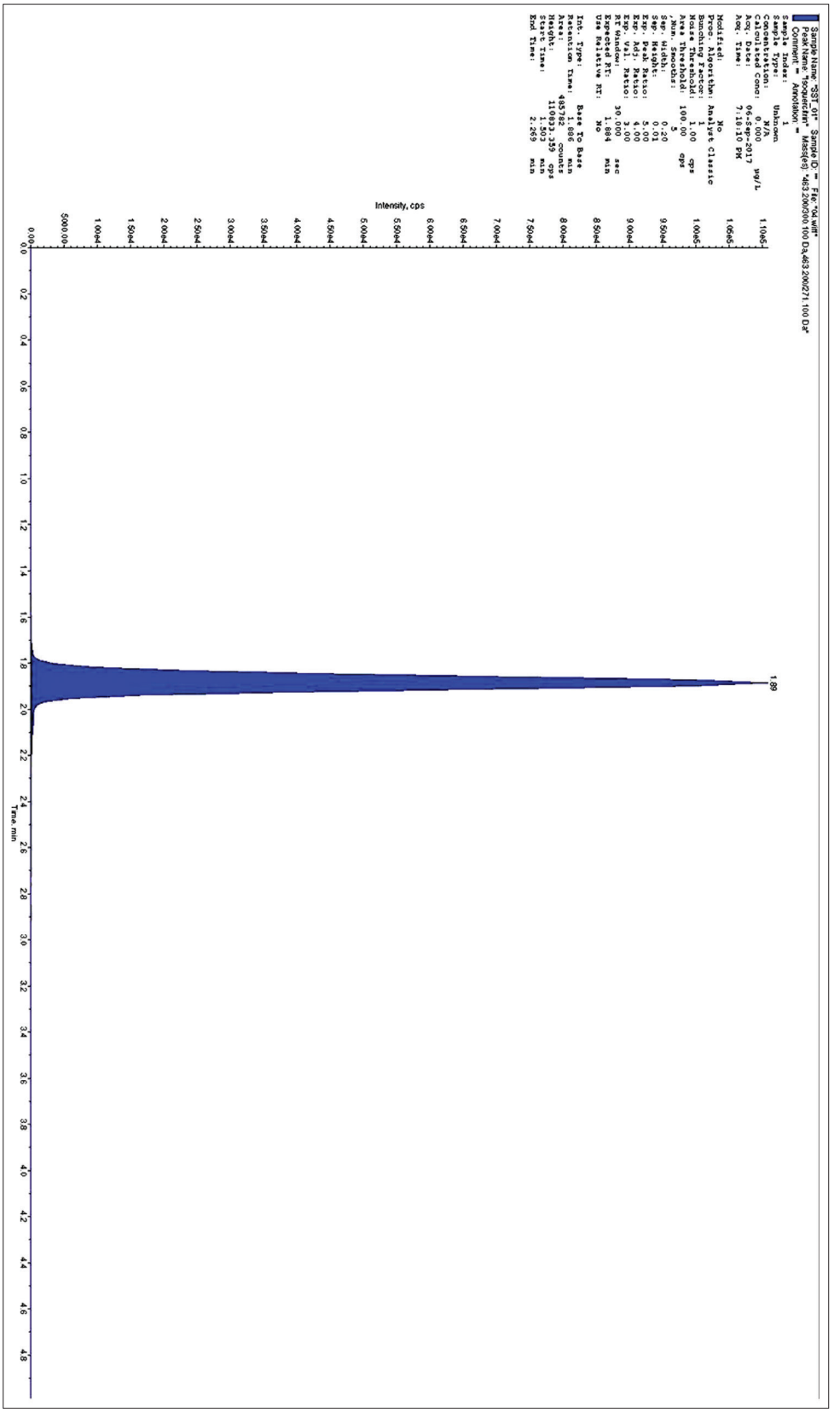

Figure 6: System Suitability Solution of Isoquercitrin.

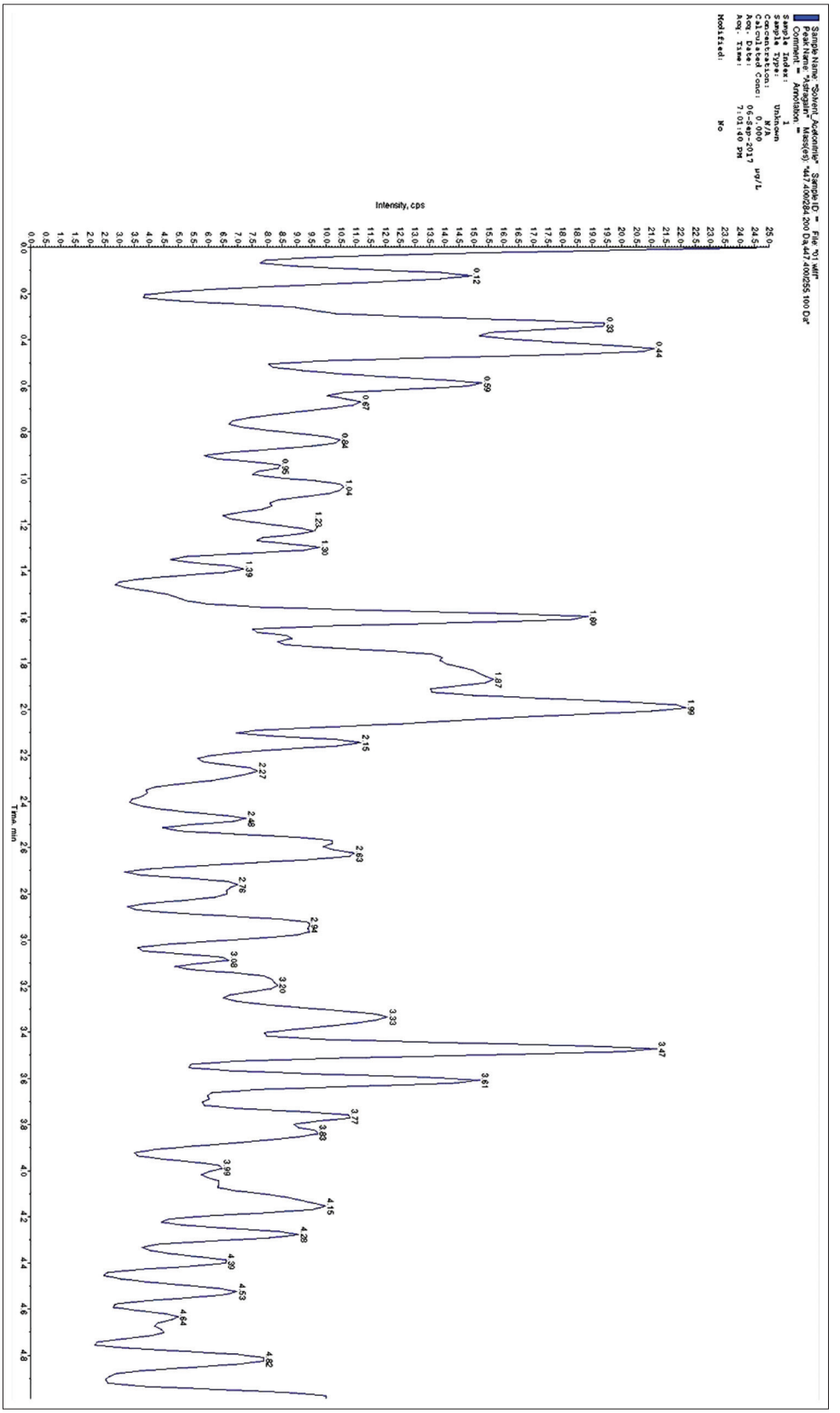

Figure 7: Solvent (Acetonitrile) of Astragalin.

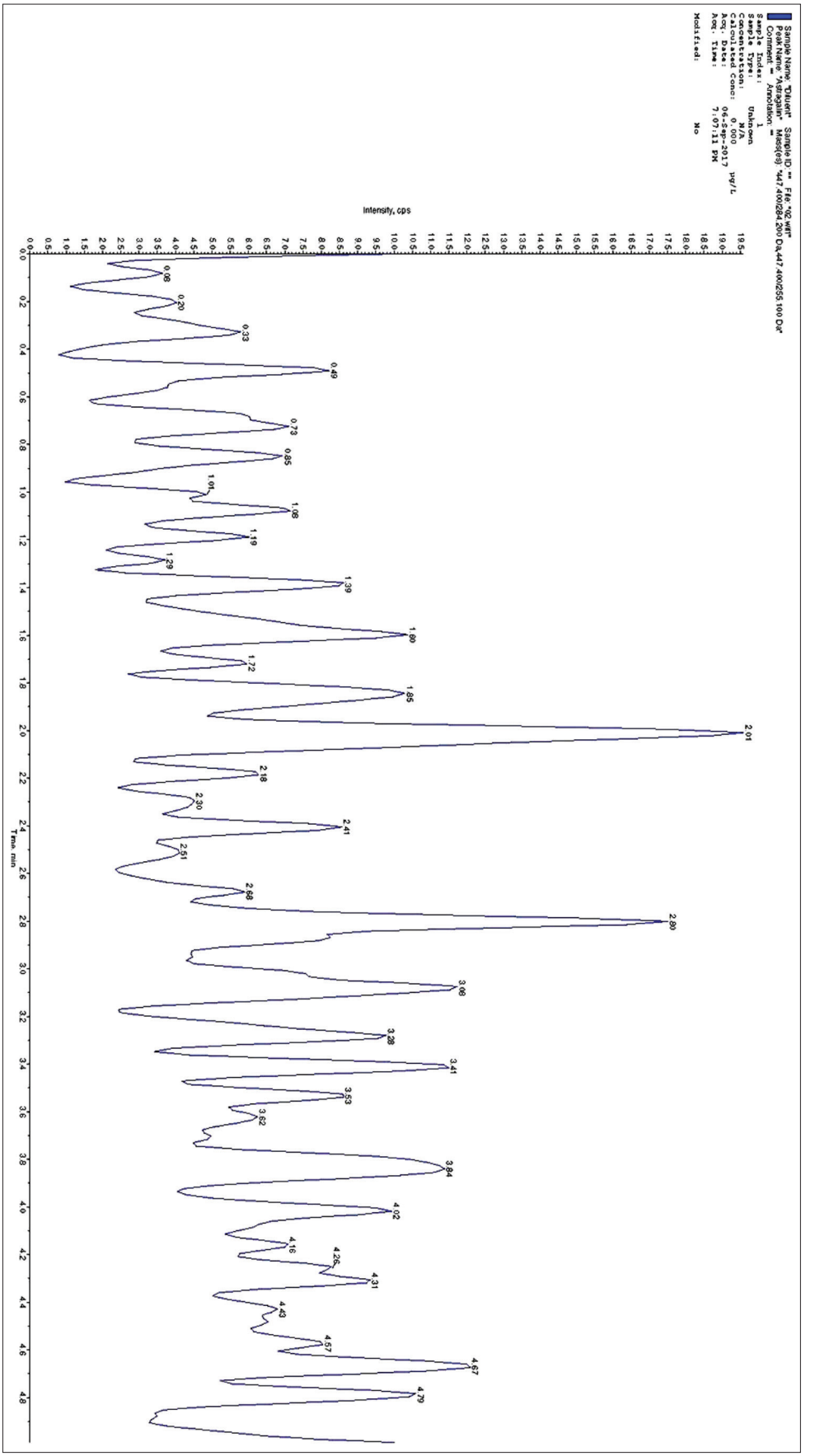

Figure 8: Diluent - (Acetonitrile: Milli-Q water (80:20), v/v) of Astragalin.

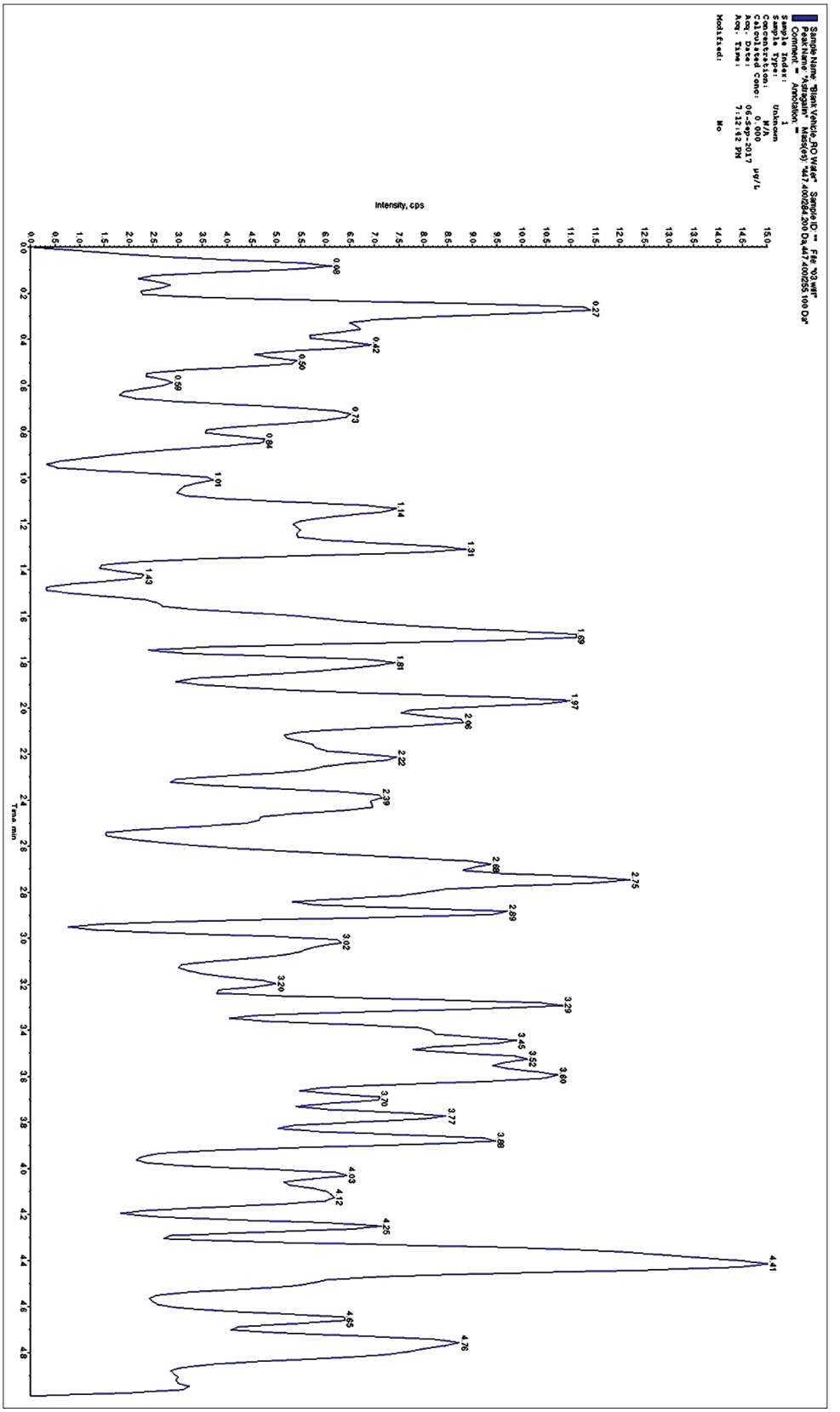

Figure 9: Blank Matrix (RO Water) of Astragalin.

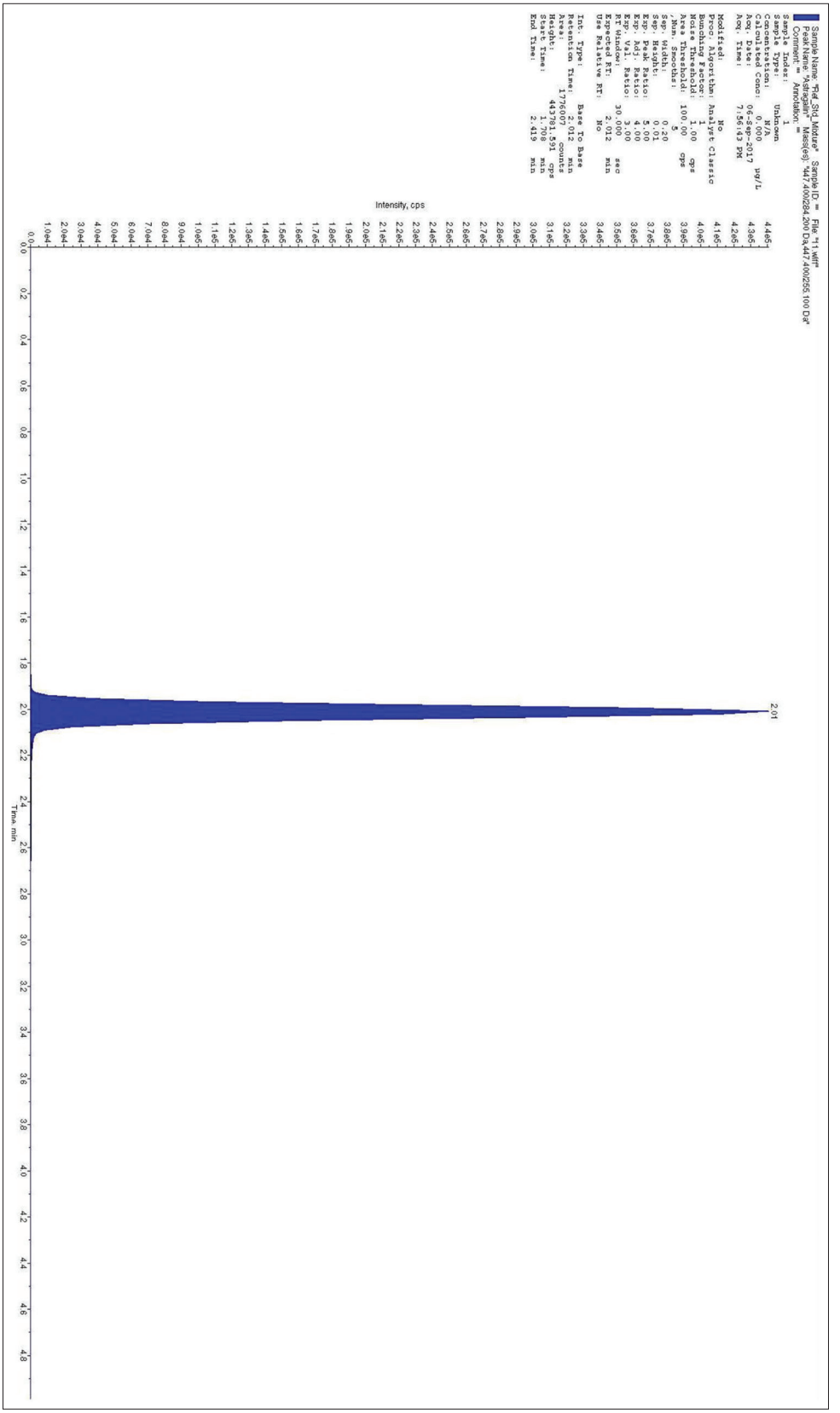

Figure 10: Reference Standard Solution of Astragalin.

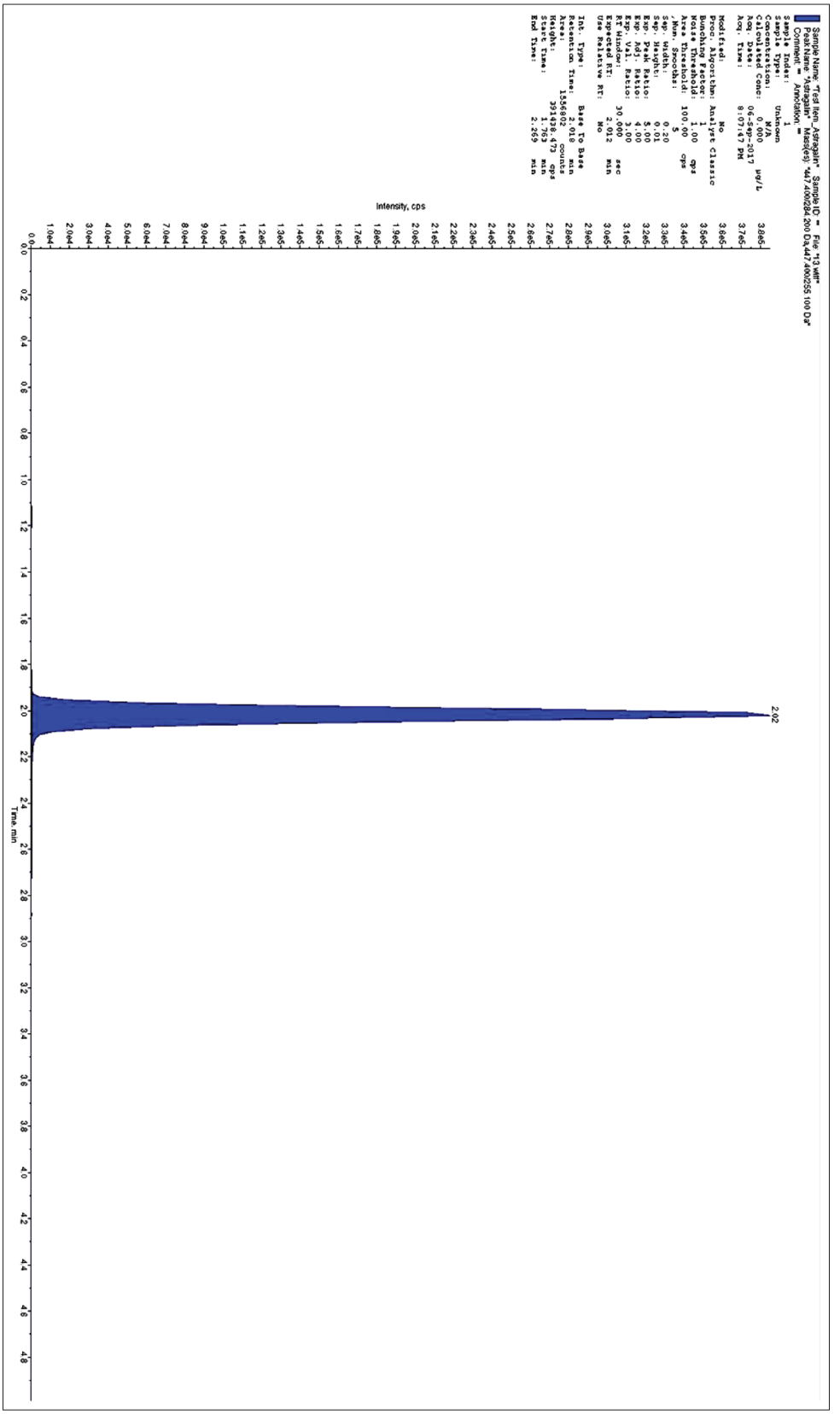

Figure 11: Test Substance Solution of Astragalin.

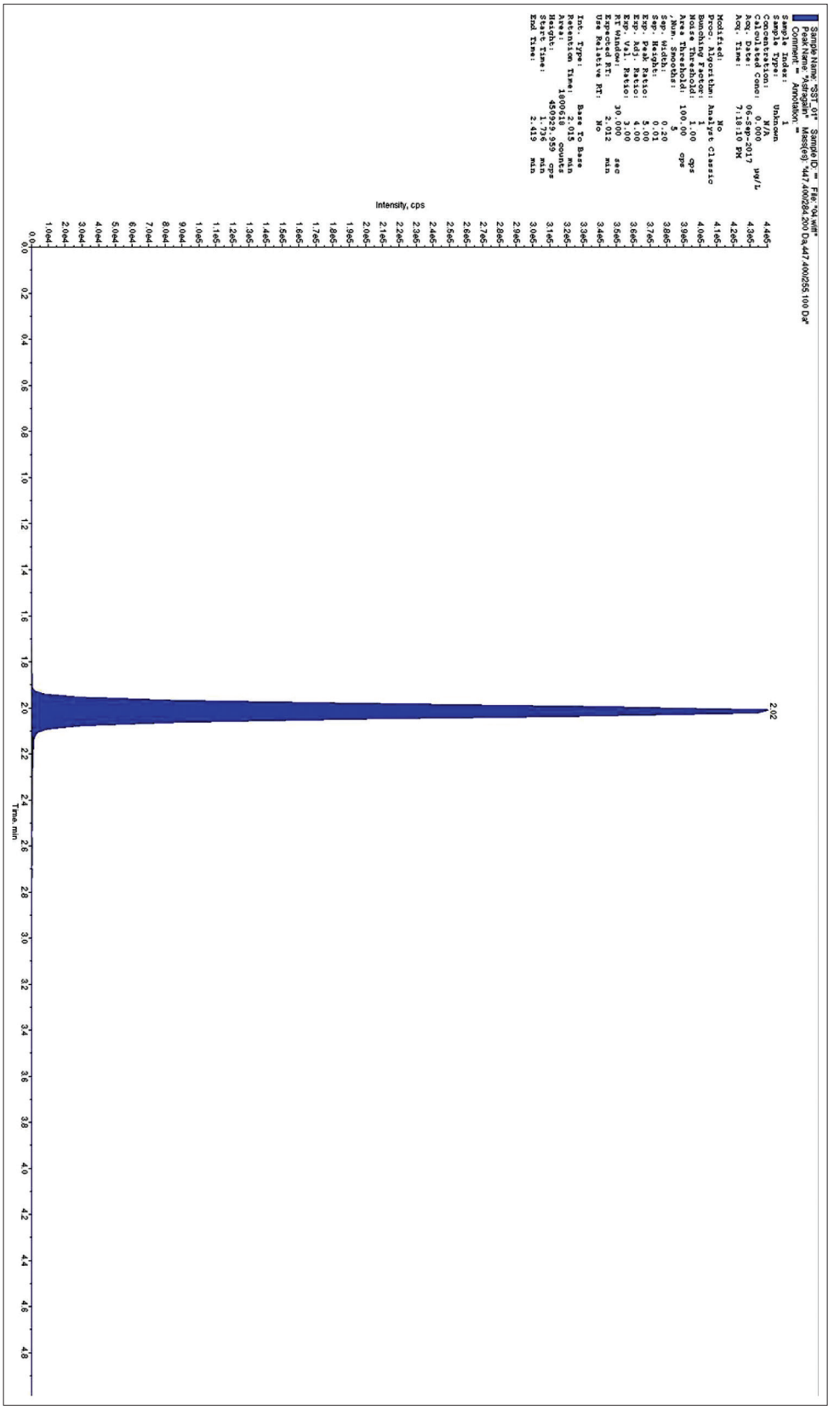

Figure 12: System Suitability Solution of Astragalin.

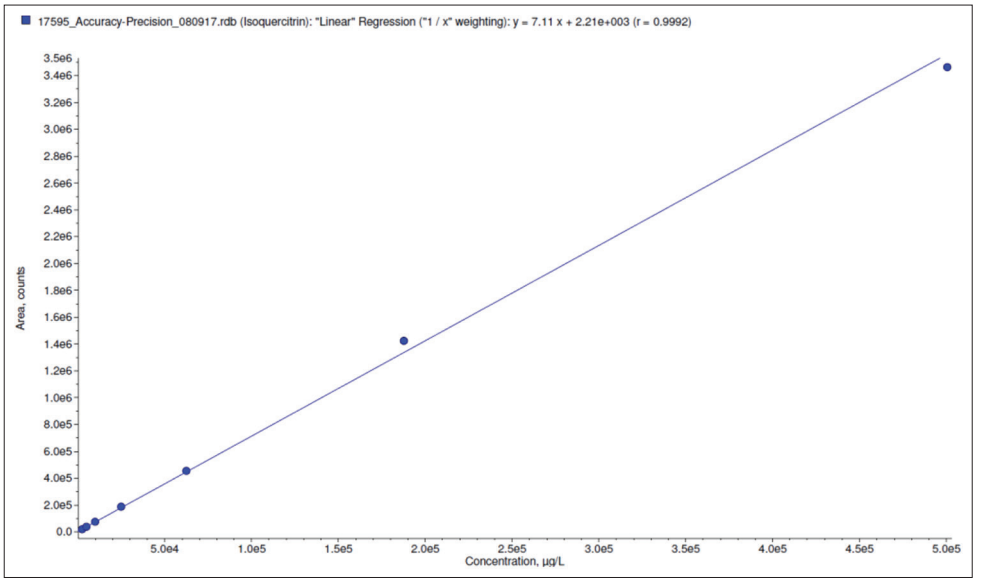

Figure 13: Linearity Curve of Reference Standard Isoquercitrin.

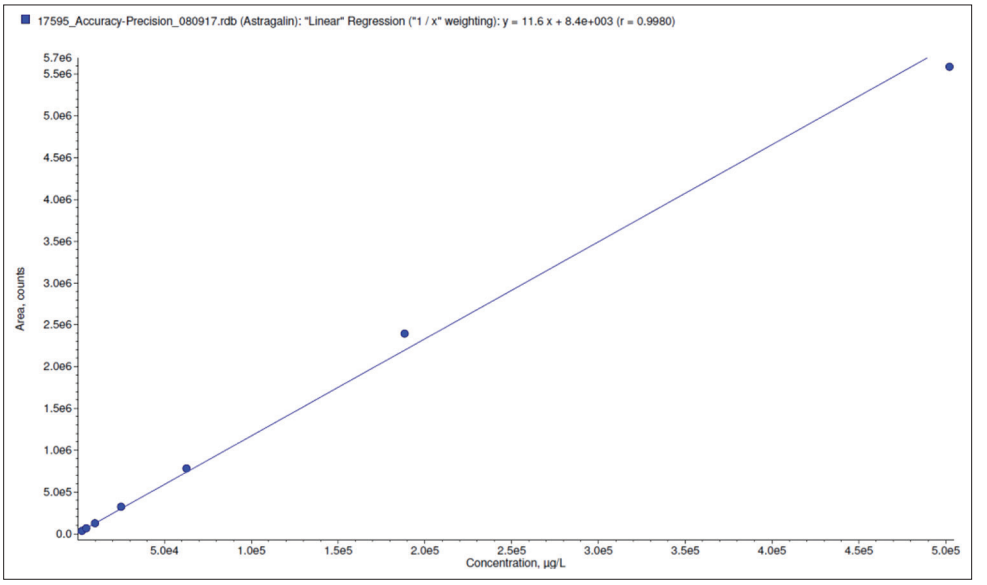

Figure 14: Linearity Curve of Reference Standard Astragalin.