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Certification of Standard Reference Material® 3389
Ginsenoside Calibration Solution

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NIST
National Institute of
Standards and Technology
U.S. Department of Commerce

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Abstract

Standard Reference Material (SRM) 3389 is intended as a calibration solution for use in the determination of ginsenosides in natural matrix samples. A unit of SRM 3389 consists of two different ampule solutions: (1) four ampules of a six-component mixture of ginsenoside Rb1, Rb2, Rc, Rd, Re, and Rg1 and (2) one ampule of a single component ginsenoside Rf. This publication documents the production, analytical methods, and statistical evaluations involved in production of this SRM.

Keywords

Ginsenoside
Liquid chromatography – mass spectrometry (LC-MS)
Liquid chromatography – ultraviolet/visible absorbance (LC-UV)
Method development
Standard Reference Material

Technical Information Contact for this SRM

Please address technical questions about this SRM to srms@nist.gov where they will be assigned to the appropriate Technical Project Leader responsible for support of this material. For sales and customer service inquiries, please contact srminfo@nist.gov.

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Table of Abbreviations

Acetonitrile	ACN
Certificate of Analysis	COA
Certified Reference Material	CRM
Chemical Sciences Division	CSD
Electrospray Ionization	ESI
Evaporative Light Scattering	ELS
Fluorescence	FL
Formic Acid	FA
Institute for National Measurement Standards	INMS
Liquid Chromatography/Mass Spectrometry	LC-MS
Liquid Chromatography/Ultraviolet-Visible Absorbance	LC-UV
Methanol	MeOH
National Institute of Standards and Technology	NIST
National Research Council Canada	NRC
Pulsed Amperometry	PA
Quantitative Proton Nuclear Magnetic Resonance Spectroscopy	q^1H-NMR
Selective Ion Monitoring	SIM
Standard Reference Material	SRM
Tandem Mass Spectrometry	MS/MS
Water	H_2O

Purpose and Description

This Standard Reference Material (SRM) is intended for use as a calibration solution for the determination of ginsenosides in natural matrix samples. A unit of SRM 3389 consists of two different ampouled methanol solutions: (1) four ampoules of a six-component mixture of ginsenoside Rb1, Rb2, Rc, Rd, Re, and Rg1 and (2) one ampoule of a single component ginsenoside Rf. This publication documents the production, analytical methods, and statistical evaluations involved in production of this SRM.

SRM Preparation

The methanol solutions of the selected ginsenosides were prepared gravimetrically by Cerilliant/PhytoLab (Round Rock, TX, USA) from individual neat compounds obtained from the National Research Council (NRC) of Canada (Ottawa, ON, Canada). Purities of the neat ginsenoside materials were assigned by the Institute for National Measurement Standards (INMS) at NRC Canada in 2011 (Appendix E). These values were assigned by quantitative proton nuclear magnetic resonance spectroscopy (q^{1}H-NMR) using the National Institute of Standards and Technology (NIST) benzoic acid SRM 350b. The SRM solution was aliquoted (1 mL) into 2 mL amber glass ampoules and flame sealed. Samples representing early, middle, and late stages of ampouling for the six-component ginsenoside solution were analyzed by Cerilliant using liquid chromatography with ultraviolet-visible absorbance detection (LC-UV). Samples for the one-component ginsenoside Rf solution were analyzed by Cerilliant using LC with mass spectrometric detection (LC-MS) and residual solvent analysis by headspace gas chromatography with flame ionization detection. No evidence of sample inhomogeneity was observed, and samples were shipped back to the Chemical Science Division (CSD) at NIST.

Storage and Use

Sealed ampoules of SRM 3389 should be stored in the dark at or below -20 °C until analysis. The ampoules should be allowed to warm to room temperature for at least 30 min. Samples of the SRM for analysis should be withdrawn from ampoules and used without delay. Certified values are not valid for ampoules which have been stored after opening, even if resealed.

Introduction

Ginsenosides are a class of triterpene saponins primarily found in the plant genus *Panax*. Ginsenosides can be isolated from different parts of the *Panax* plant, but the root is typically the primary source. More than 150 naturally occurring ginsenosides have been isolated from roots, leaves, stems, fruits, and/or flower heads of *Panax* genus. Due to their unique physiological activity, the separation and determination of each ginsenoside is required. The majority of these ginsenosides are classified as part of the dammarane family. These ginsenosides consist of a four-ring steroid aglycone main structure with multiple available hydroxyl groups, which are glycosylated with one or two carbohydrate units. Ginsenosides have been reported to have a wide range of biologically activities such as neuroprotective [1-4], anti-cancer [5, 6], cardio protective [7], anti-diabetic [8], antinociception [9], anti-oxidant [10-12], vasodilating [13], and hepatoprotective effects [14].

LC is the most prevalent separation technique for mixtures of ginsenosides because of its speed and selectivity. LC has the additional advantage of compatibility with various detection techniques

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such as UV absorbance [15-19], evaporative light scattering (ELS) [20], fluorescence (FL) [21], pulsed amperometry (PA) [22], MS [15, 23, 24], and tandem mass spectrometry (MS/MS) [25-27]. UV is the most frequently used detector for simple sample matrixes because of its inexpensive cost and simplicity, but ginsenoside detection is limited to short wavelengths of 200 nm to 205 nm. Mass spectrometry provides a good alternative detection technique to UV for ginsenosides as they lack chromophores that span broad wavelengths.

Experimental Methods

The two analytical methods used in the certification of SRM 3389 were LC-UV and LC-MS, which are described in detail in Appendix A and Appendix B, respectively.

Certification Measurement Results

The certification measurements and statistical analysis are described in detail in Appendix C and Appendix D, respectively.

Certified Values
The certified mass fraction values for ginsenosides Rb1, Rb2, Rc, Rd, Re, and Rg1 are shown in Table 1. A NIST certified value is a value for which NIST has the highest confidence in that all known or suspected sources of bias have been accounted for.

Table 1. Mass-fraction values for the six-component solution ($k = 2.0$)

Ginsenoside	Mass Fraction $x \pm U_{95\%}$ (mg/g)
Rg1	0.524 ± 0.012
Re	1.55 ± 0.03
Rb1	3.83 ± 0.12
Rc	0.754 ± 0.020
Rb2	0.889 ± 0.028
Rd	0.248 ± 0.008

Uncertainty Statement for Certified Values
Mass fraction values are expressed as $x \pm U_{95\%}(x)$, where x is the certified value and $U_{95\%}(x)$ is the expanded uncertainty of the certified value. The true value of the analyte is believed to lie within the interval $x \pm U_{95\%}(x)$ with 95 % confidence. To propagate this uncertainty, treat the certified value as a normally distributed random variable with mean x and standard deviation $U_{95\%}(x)/2$. The number of significant figures for the certified values were determined based on the number of significant figures for the purity of neat individual reference standards used as calibrants in the measurements. The number of decimal places for the uncertainties are based on the certified value.

Traceability Statement for Certified Values
Metrological traceability is to the measurement units realized through purity determinations of the primary standards of the INMS at NRC Canada.

Non-Certified Values

The non-certified mass fraction values for ginsenoside Rf is shown in Table 2. The mass fraction value for Rf in the single-component solution does not meet NIST's criteria for certification. This value is, however, the best currently available estimate.

Table 2. Mass-fraction for the single-component solution ($k = 2.0$)

Ginsenoside	Mass Fraction $x \pm U_{95\%}$ (mg/g)
Rf	1.3 ± 0.1

Uncertainty Statement for Non-Certified Values

The value is expressed as $x \pm U_{95\%}(x)$, where x is the value and $U_{95\%}(x)$ is the expanded uncertainty of the value. The true value of the analyte is believed to lie within the interval $x \pm U_{95\%}(x)$ with about 95 % confidence. To propagate this uncertainty, treat the value as a normally distributed random variable with mean x and standard deviation $U_{95\%}(x)/2$. The number of significant figures for the non-certified value was determined based on the number of significant figures for the purity of neat individual reference standards used as calibrants in the measurements. The number of decimal places for the uncertainty is based on the non-certified value.

Traceability Statement for Non-Certified Values

Metrological traceability is through the LC-UV and LC-MS methods used for value assignment.

Certificate of Analysis

In accordance with ISO Guide 31: 2000, a NIST SRM certificate is a document containing the name, description, and intended purpose of the material, the logo of the U.S. Department of Commerce, the name of NIST as a certifying body, instructions for proper use and storage of the material, certified property value(s) with associated uncertainty(ies), method(s) used to obtain property values, the period of validity, if appropriate, and any other technical information deemed necessary for its proper use. A Certificate is issued for an SRM certified for one or more specific physical or engineering performance properties and may contain NIST reference, information, or both values in addition to certified values. A Certificate of Analysis is issued for an SRM certified for one or more specific chemical properties. Note: ISO Guide 31 is updated periodically; check with ISO for the latest version. [https://www.nist.gov/srm/srm-definitions]

For the most current version of the Certificate of Analysis (COA) for NIST SRM 3389 Ginsenoside Calibration Solution, please visit: https://www-s.nist.gov/srmors/viewdetail.cfm?srm=3389.

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Appendix A

Method Development for the Separation of 12 Ginsenosides in Solution via LC-UV

A. Materials

A total of 12 ginsenoside reference standards were obtained from Cerilliant. The impurities in this material were previously evaluated by PhytoLab, the supplier of the material to Cerilliant. The CAS numbers, molecular formula, molar mass, batch numbers, and purity of the 12 ginsenosides are listed in Table A-1. Certified Reference Material (CRM) MIGS-1 (Ginsenoside Mixture) was obtained from the NRC of Canada (Ottawa, Ontario, Canada). SRM 3389 preparation is described in detail previously on page 9. HPLC grade acetonitrile (ACN), methanol (MeOH), and water (H_2O) were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Table A-1. Ginsenoside reference standards from PhytoLab

Ginsenoside	CAS #	Molecular Formula	Molar Mass (g/mol)	Batch #	Purity[a] (%)
Rb1	41753-43-9	$C_{54}H_{92}O_{23}$	1109.31	2461	91
Rb2	11021-13-9	$C_{53}H_{90}O_{22}$	1079.29	5247	82
Rb3	68406-26-8	$C_{53}H_{90}O_{22}$	1079.29	5008	91
Rc	11021-14-0	$C_{53}H_{90}O_{22}$	1079.29	5677	87
Rd	52705-93-8	$C_{48}H_{82}O_{18}$	1079.29	5678	93
Re	52286-59-6	$C_{48}H_{82}O_{18}$	947.17	3180	87
Rf	52286-58-5	$C_{42}H_{72}O_{14}$	801.03	10535	94
Rg1	22427-39-0	$C_{42}H_{72}O_{14}$	801.03	3013	91
Rg2	52286-74-5	$C_{42}H_{72}O_{13}$	785.03	11314	94
Rg3	14197-60-5	$C_{42}H_{72}O_{13}$	785.03	2460	93
Rh1	63223-86-9	$C_{36}H_{62}O_9$	638.89	3218	93
Rh2	78214-33-2	$C_{36}H_{62}O_8$	622.89	5010	85

[a] Chromatographic purity values assigned by the manufacturer based on LC-UV measurements.

A. Sample Preparation

Small portions of each compound were dissolved in 1 mL aliquots of MeOH. The mixture of the 12 ginsenosides was prepared by combining small quantities (< 100 µL) of each ginsenoside. For preliminary evaluation, a single ampule of the CRM MIGS-1 and SRM 3389 was removed from the freezer and the solutions were transferred to individual LC autosampler vial for analysis.

A. Instrumental Method

The analyses were performed on an Dionex Ultimate 3000 LC system equipped with a pump, autosampler, column compartment, and diode array detector. The instrument was computer controlled using commercial software (Chromeleon v. 6.8, Thermo Scientific). Separations were carried out on a octadecyl (C_{18}) column (ACE 3 C_{18}) purchased from Advanced Chromatography Technologies (ACE, Aberdeen, Scotland) with the following characteristics: 15 cm length, 4.6 mm diameter, and 3 µm average particle diameters. The chromatographic conditions for the separation of the 12 ginsenosides are discussed in later sections. All sample injections were held constant at 2 µL.

A. Results and Discussion

The goal of this project was to develop a new multi-component LC-UV method for the separation of the seven ginsenosides present in SRM 3389 and five additional ginsenosides that have been detected in matrix-based materials such as ground root and root extract. MacCrehan and White [A1] previously used an ACE 3 C_{18} (150 mm x 4.6 mm) with a 3 µm particle size to separate a "Ginseng Ginsenosides Mix" from Cerilliant. Baseline separation was obtained for all ginsenosides within 65 min using an ACN and H_2O mobile phase gradient.

For the current study, the mobile phase system was modified (Table A-2) to separate a mixture 12 ginsenosides. An optimized separation of the 12 ginsenosides is shown in Figure A-1 using the mobile phase gradient in Table A-2. In addition, the flowrate, column temperature, and absorbance wavelength were held constant at 0.7 mL/min, 25 $^{\circ}$C, and 200 nm, respectively.

Table A-2. Mobile phase gradient for the LC-UV

Time (min)	H_2O (%)	ACN (%)
-5.0	78	22
0.0	78	22
10.0	78	22
29.0	58	42
59.0	0	100
60.0	0	100
60.1	78	22

Using these conditions, all the ginsenoside components present in the CRM MIGS-1 and SRM 3389 were baseline resolved. The complete separation of these ginsenosides are required for the LC-UV certification of SRM 3389, which uses CRM MIGS-1 as a control. Several key parameters including flowrate, mobile phase hold time, and column temperature are discussed below. Ginsenoside Rg3 and Rh2 were not included in these studies because they are baseline resolved and elute ≈ 8 min and ≈ 15 min later in the chromatogram, respectively.

In LC, the mobile phase flowrate influences separation efficiency and detection sensitivity. Increased flow produces narrower peaks and higher signal response from the detector. However, separation efficiency is reduced at high linear velocities since mass transfer is limited (*i.e.*, the C term of the van Deemter equation becomes more significant). At very low linear velocities, diffusion can decrease separation efficiency, although in practice this condition is rarely significant in modern LC. The appropriate flowrate is typically based on the dimensions of the column and pressure limitation of the LC system to provide the highest efficiency possible while obtaining good chromatographic resolution.

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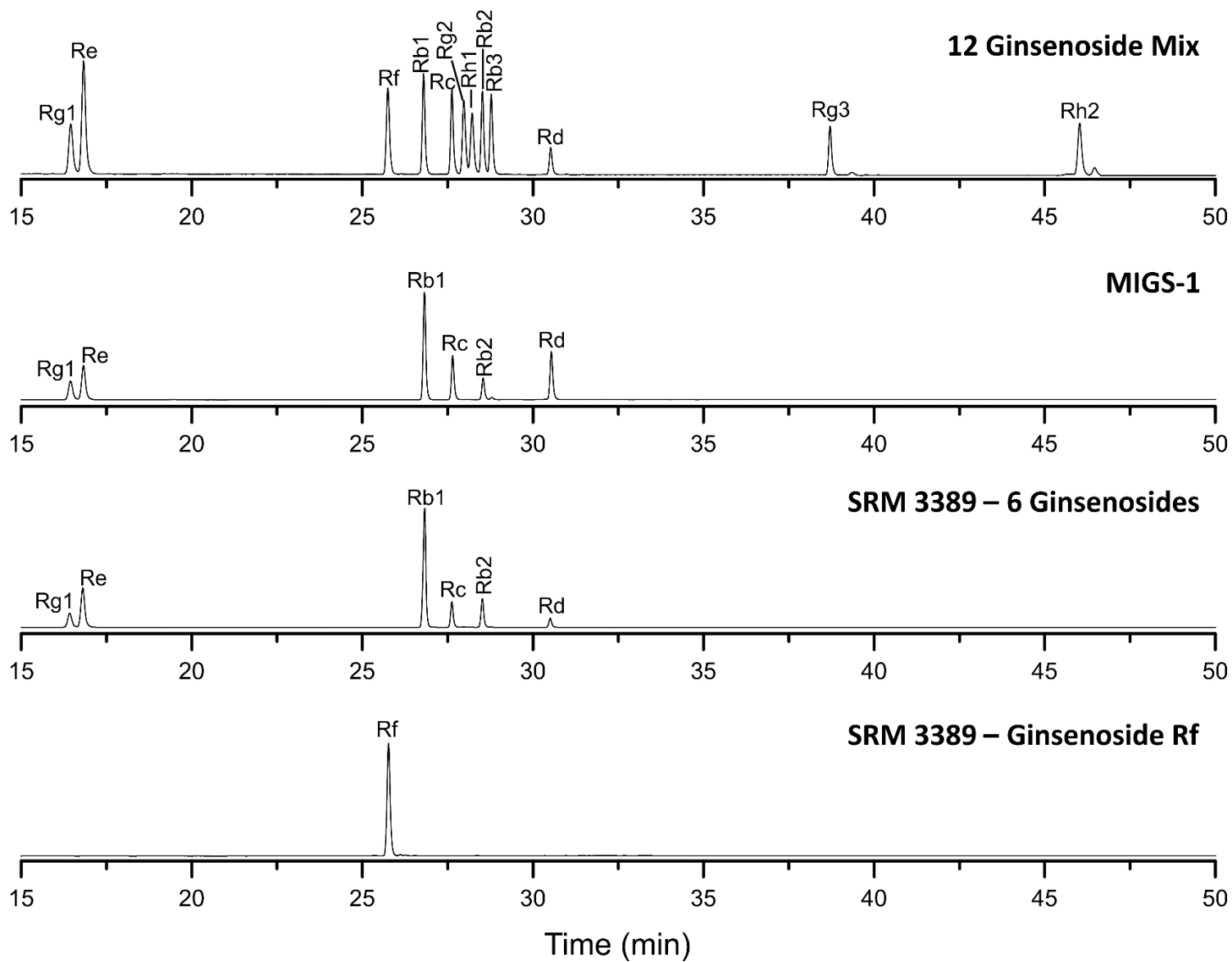
Figure A-1. LC separations under optimized ACN mobile phase gradient

Column selectivity is normally attributed to properties of the stationary phase and mobile phase composition and is not affected by the flowrate. For this reason, it was surprising to observe a significant difference in the retention behavior of two ginsenosides at different flowrates as illustrated in Figure A-2. As the flowrate increased from 0.6 mL/min to 1.0 mL/min, the retention of Rg2 (6) and Rh1 (7) unexpectedly decreased. The ginsenosides have similar molecular structures; but differ in the number of carbohydrate units attached at the same hydroxyl position. The structure of Rf is similar to Rg2, differing by the addition of an ethanol group on the carbohydrate unit. However, changes in selectivity as a function of flowrate were not observed for ginsenoside Rf. The best chromatographic resolution was obtained with a flowrate of 0.7 mL/min and was chosen as the optimal flowrate.
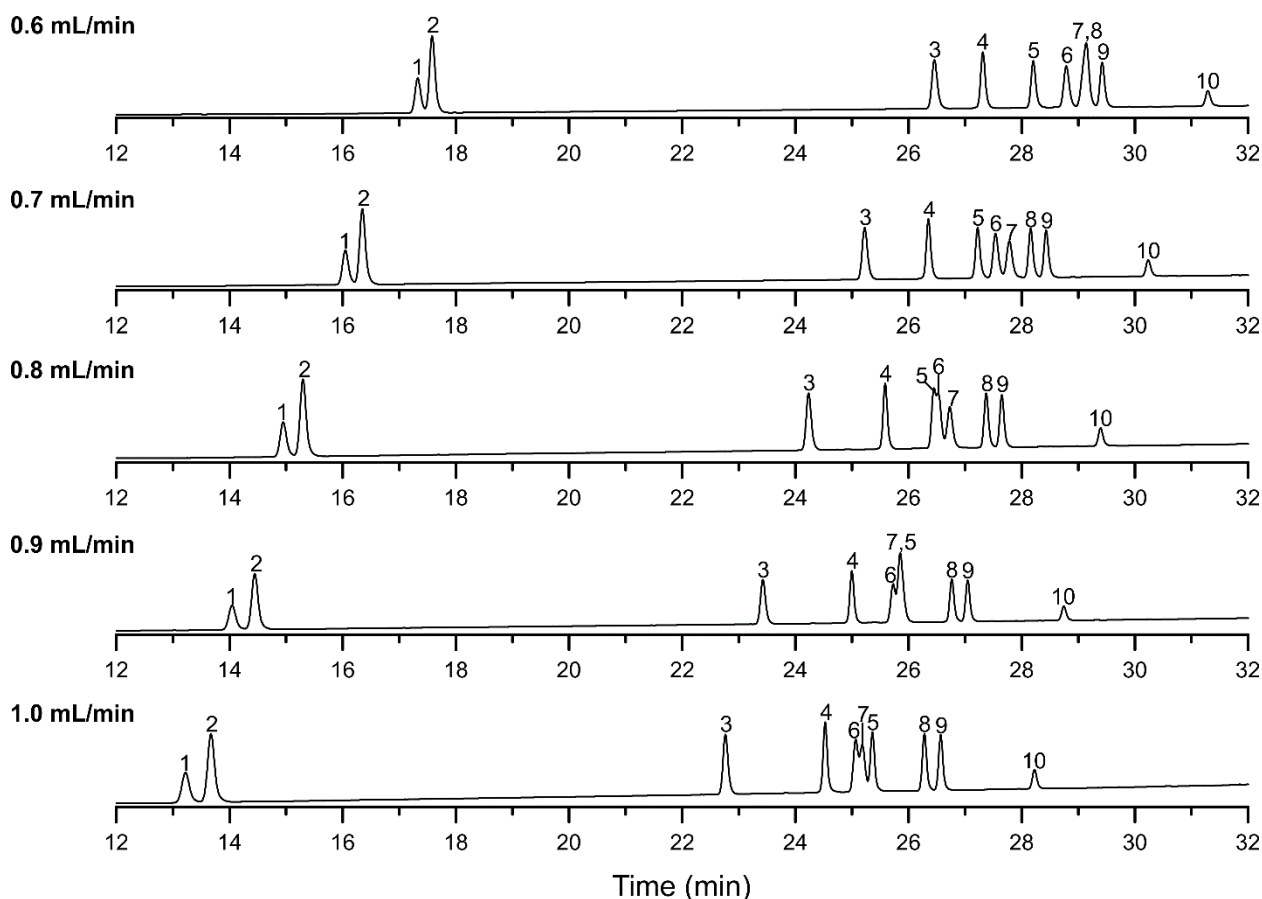
Figure A-2. LC separations using different mobile phase flowrates

The ACE 3 C_{18} column temperature and initial mobile phase composition was 25 °C and 22 % ACN, respectively. Peak identification is the following: (1) Rg1, (2) Re, (3) Rf, (4) Rb1, (5) Rc, (6) Rg2, (7) Rh1, (8) Rb2, (9) Rb3, and (10) Rd.

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The separation of the ginsenoside mixture was highly sensitive to the initial solvent conditions prior to the mobile phase gradient as shown in Figure A-3. The initial mobile phase composition was adjusted from 19 to 24 % ACN. The flowrate and column temperature were held constant at 0.7 mL/min and 25 °C, respectively. In the case of ginsenoside Rg1 and Re, retention and chromatographic resolution improved with increased ACN. The separation of ginsenoside Rf, Rb1, Rc, Rb2, Rb3, Rd, Rg3, and Rh2 were influenced less by slight mobile phase modification. However, Rg2 and Rh1 elute earlier on the C_{18} phase as the percentage of ACN increased.
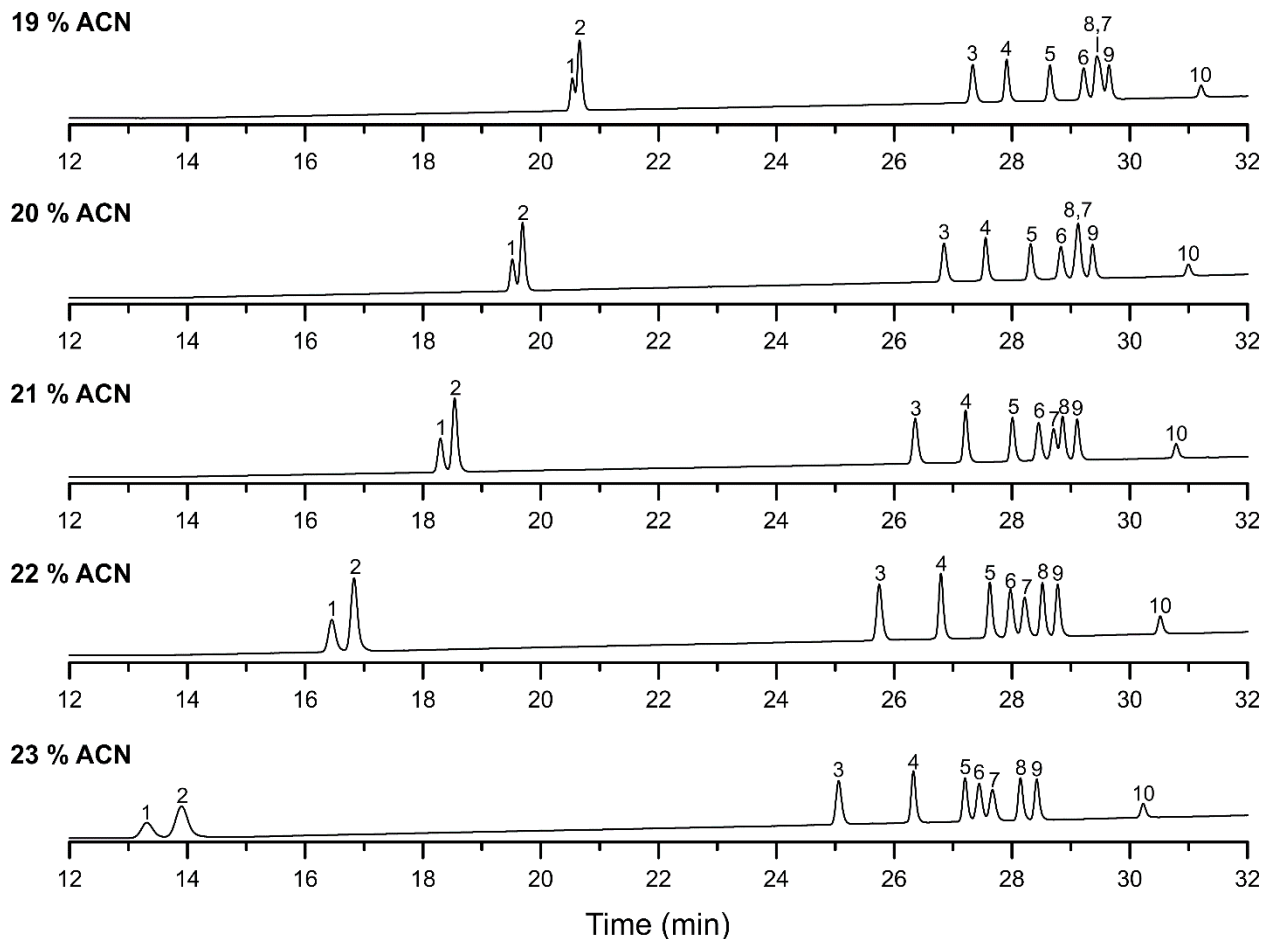
Figure A-3. LC separations using different initial isocratic conditions

The ACE 3 C_{18} column temperature and mobile phase flowrate was 25 °C and 0.7 mL/min, respectively. Peak identification is the following: (1) Rg1, (2) Re, (3) Rf, (4) Rb1, (5) Rc, (6) Rg2, (7) Rh1, (8) Rb2, (9) Rb3, and (10) Rd.

The effect of column temperature on the separation of the ginsenoside mixture is shown in Figure A-4. The column temperatures investigated in this study were 15 °C, 20 °C, 25 °C, 30 °C, and 35 °C. The flowrate and initial mobile phase were held constant at 0.70 mL/min and 22% ACN, respectively. The chromatographic resolution between Rg1 and Re decreased as the temperature increased. The retention times of the remaining ginsenosides increased with increases in the column temperature, but Rg2 and Rh1 were affected to a lesser degree.
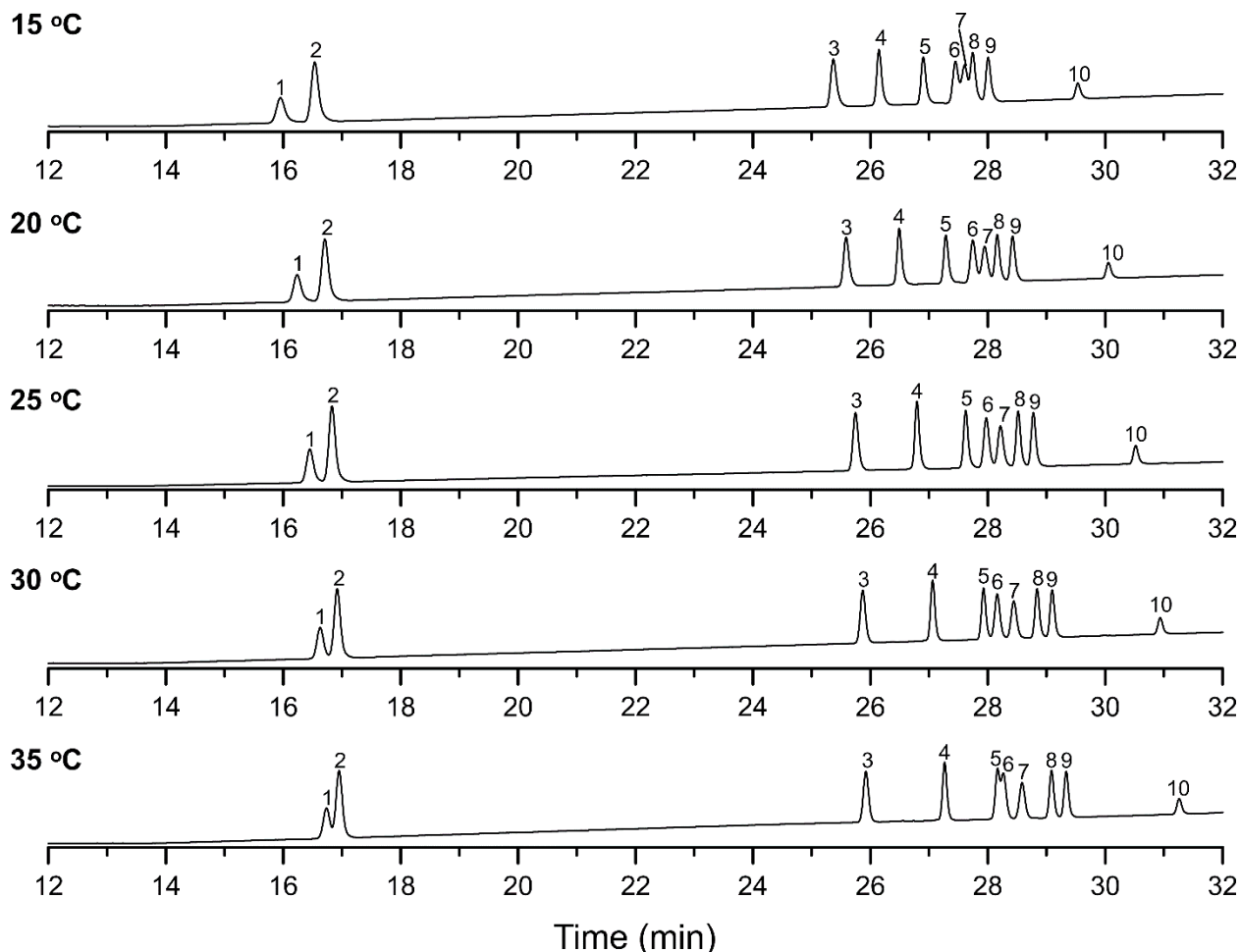
Figure A-4. LC separations using different column temperatures

The mobile phase flowrate and initial composition was 0.7 mL/min and 22 % ACN, respectively. Peak identification is the following: (1) Rg1, (2) Re, (3) Rf, (4) Rb1, (5) Rc, (6) Rg2, (7) Rh1, (8) Rb2, (9) Rb3, and (10) Rd.

A. Conclusions

An LC-UV method was developed to separate a mixture of 12 ginsenosides. The flowrate, initial mobile phase composition, and column temperature were evaluated to provide the best separation. In the case of Rg2 and Rh1, retention was strongly affected by these conditions. The developed LC-UV method will be used to help certify the mass fractions of SRM 3389 ginsenoside calibration solution.

A. References

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Appendix B

Method Development for the Separation of 12 Ginsenoside Standards via LC-MS

B. Materials

The 12 ginsenoside reference standards were obtained from Cerilliant. The impurities in this material were previously evaluated by PhytoLab, the supplier of the material to Cerilliant (Table A-1). CRM MIGS-1 (Ginsenoside Mixture) was obtained from the NRC of Canada. SRM 3389 preparation is described in detail previously. HPLC grade ACN, MeOH, and 0.1 % formic acid (FA) in H_2O were purchased from Fisher Scientific.

B. Sample Preparation

Small portions of each compound were dissolved in 1 mL aliquots of MeOH. The mixture of the 12 ginsenosides was prepared by combining small quantities (< 100 µL) of each ginsenoside solution.

B. Instrumental Method

The analyses were performed on an Agilent 1100 LC system equipped with a binary pump, degasser, autosampler, column compartment, variable wavelength absorbance detector, and a electrospray ionization (ESI-) MS. The instrument was computer controlled using commercial software (ChemStation, Agilent). The LC-MS columns and conditions for the separation of the 12 ginsenosides are discussed in later sections. All sample injections were held constant at 2 µL.

B. Results and Discussion

Mass Spectra of Ginsenosides

In LC-MS, the choice between positive and negative ion modes for ESI-MS has been shown to play a significant role in the detection sensitivity of ginsenosides [B1-B3]. Based on previous work by Miao et al. [B4], negative ion mode was shown to provide higher sensitivity, but positive mode provided more structural information. Negative mode was chosen in these studies because the collection of structural information was not a priority. The ESI-MS of the 12 ginsenosides are shown in Figure B-1 to Figure B-12. The peak distribution ($\geq 10\%$) is summarized in Table B-1. The mass spectra were collected using the following parameters: (1) capillary potential of 4.0 kV; (2) electrospray source temperature of 200 °C; (3) desolvation gas temperature of 250 °C; and (4) cone voltage of 70 V. No attempts were made to optimize these parameters.

The maximum signal abundance was observed for the formation of the FA adduct ion, which is represented by the singly charged ion $[M - 2H + HCO_2H]^-$ except for ginsenosides Rc and Rb1. Doubly charged ion, $[M - 2H]^{2-}$, was observed in the mass spectra of ginsenosides Rc, Rd, Re, Rf, Rb1, Rb2, Rb3, Rc, Rg2, and Rg3. The focus was to determine the appropriate ion to use in the SIM mode for future LC-MS measurements and to allow for chemical identity. The ion peaks with the maximum signal abundance reported in Table B-1 were chosen for these studies except for ginsenoside Rc (*m/z* 1123.5).

Table B-1. Peak distribution in the negative ion mode mass spectra

Ginsenoside	Molecular Formula	Molar Mass (g/mol)	m/z [a]
Rb1	$C_{54}H_{92}O_{23}$	1109.31	<u>1107.5</u>, 1108.5, 1109.5, 1153.5, 1154.5
Rb2	$C_{53}H_{90}O_{22}$	1079.29	829.5, 1077.4, 1078.4, 1079.4, <u>1123.5</u>, 1191.4, 1192.4
Rb3	$C_{53}H_{90}O_{22}$	1079.29	1077.5, 1078.5, 1079.5, <u>1123.5</u>, 1124.5, 1125.5, 1191.3
Rc	$C_{53}H_{90}O_{22}$	1079.29	829.4, 830.4, <u>1077.5</u>, 1078.5, 1079.5, 1123.5, 1124.5, 1191.6
Rd	$C_{48}H_{82}O_{18}$	947.17	945.6, <u>991.7</u>, 992.7, 1059.5, 1060.5
Re	$C_{48}H_{82}O_{18}$	947.17	<u>991.7</u>, 992.7, 993.7, 1059.5
Rf	$C_{42}H_{72}O_{14}$	801.03	799.3, 800.3, <u>845.5</u>, 846.5, 847.5, 913.4
Rg1	$C_{42}H_{72}O_{14}$	801.03	<u>845.4</u>, 846.4
Rg2	$C_{42}H_{72}O_{13}$	785.03	<u>829.5</u>, 830.5, 831.5, 897
Rg3	$C_{42}H_{72}O_{13}$	785.03	783.4, <u>829.4</u>, 830.4, 831.4, 897.3
Rh1	$C_{36}H_{62}O_{9}$	638.89	<u>683.4</u>, 684.4
Rh2	$C_{36}H_{62}O_{8}$	622.89	<u>667.4</u>, 668.4, 669.4

[a] All m/z peaks had relative abundance values greater than 10%.
[b] The underlined m/z represents the most abundant peak signal in the mass spectra
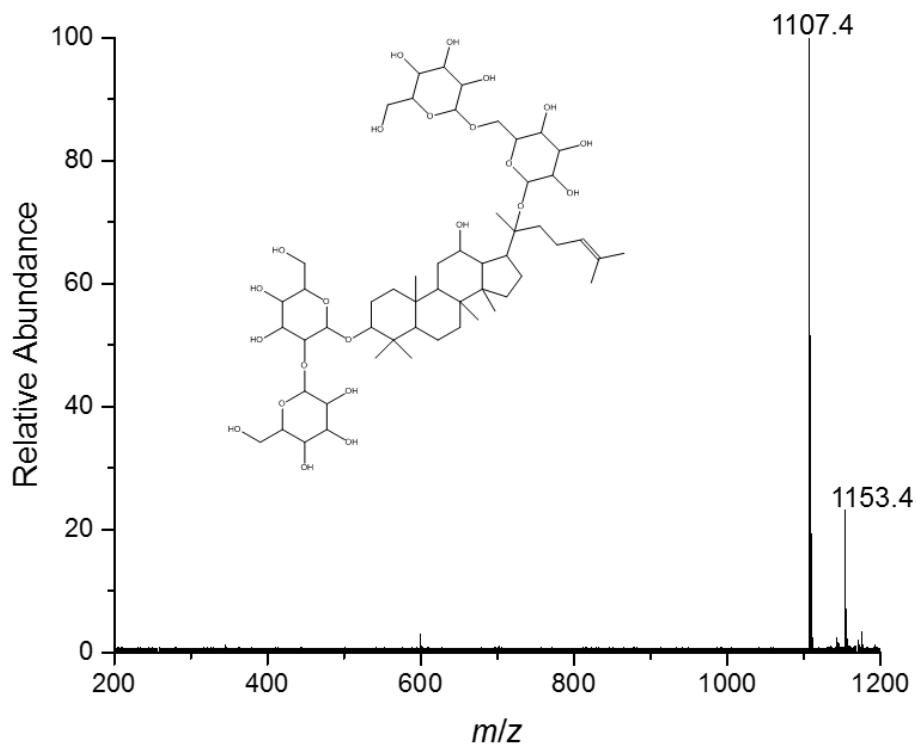
Figure B-1. Mass spectra and molecular structure of ginsenoside Rb1 (1109.31 g/mol)
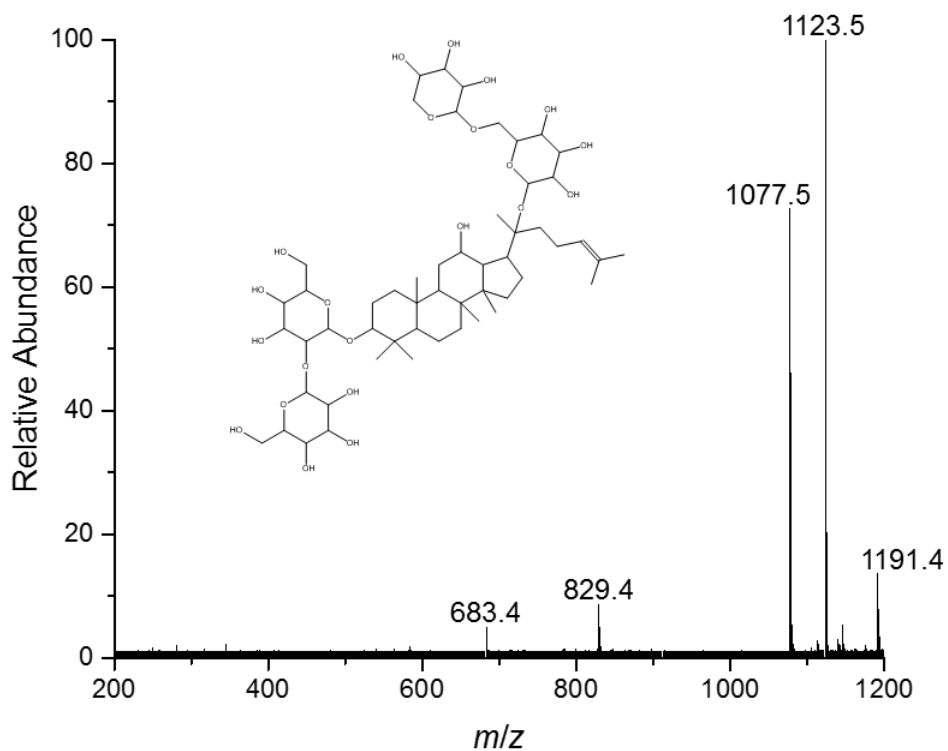
Figure B-2. Mass spectra and molecular structure of ginsenoside Rb2 (1079.29 g/mol)
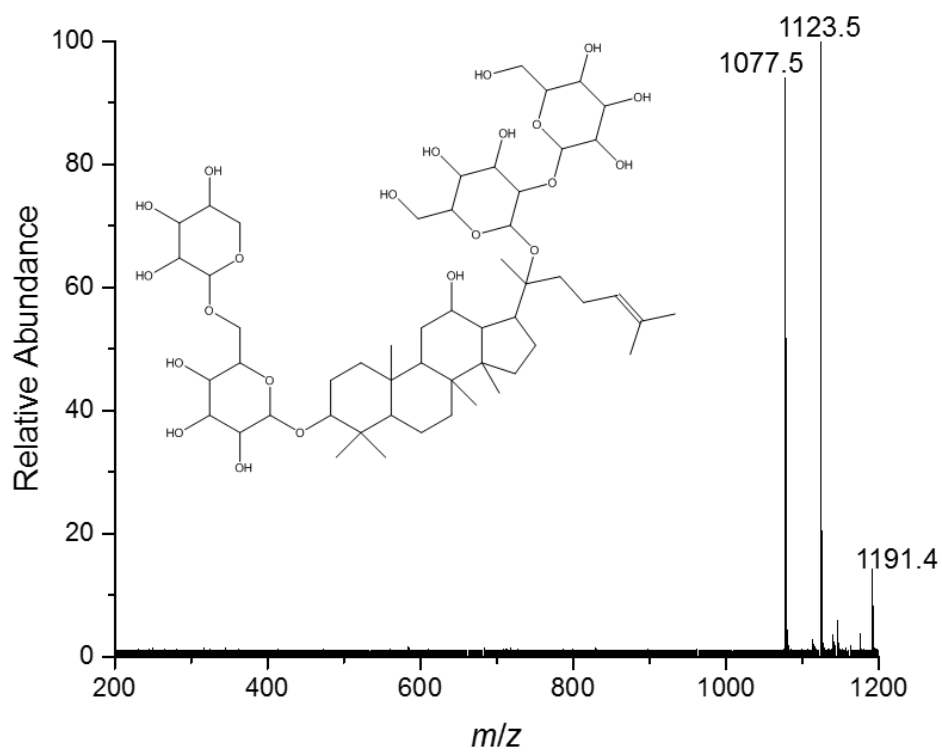
Figure B-3. Mass spectra and molecular structure of ginsenoside Rb3 (1079.29 g/mol)
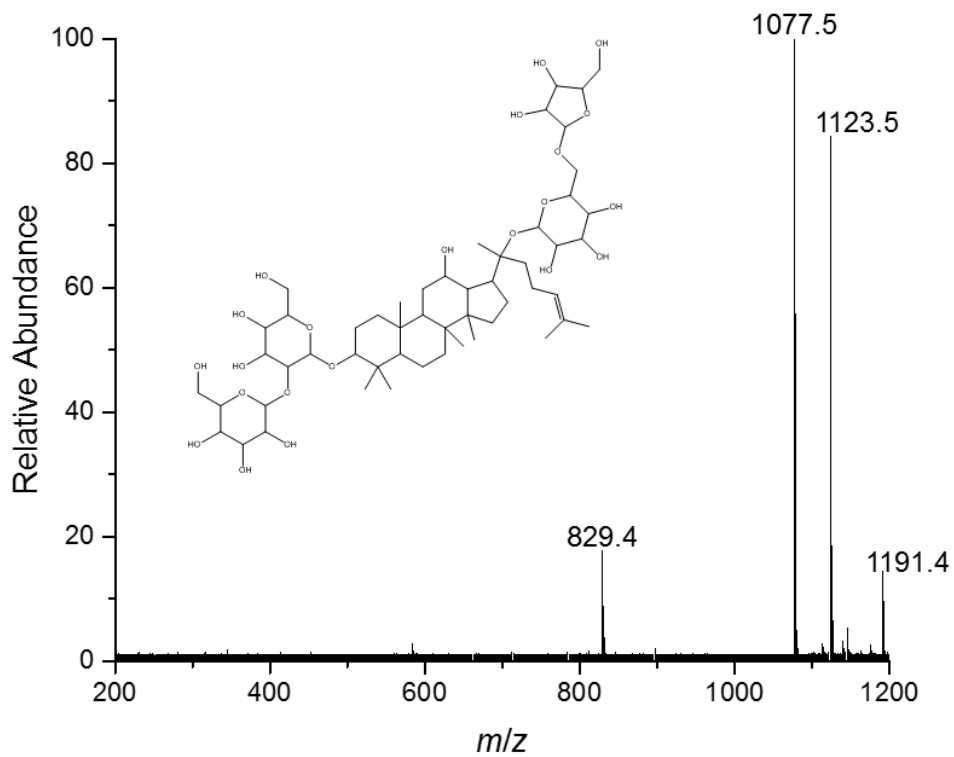
Figure B-4. Mass spectra and molecular structure of ginsenoside Rc (1079.29 g/mol)
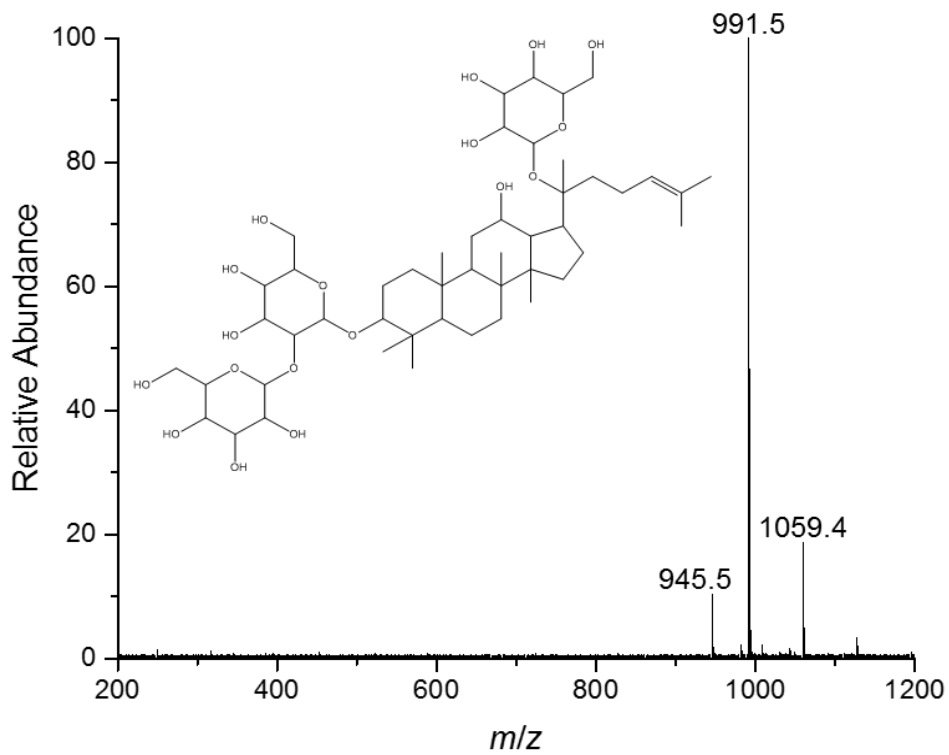
Figure B-5. Mass spectra and molecular structure of ginsenoside Rd (1079.29 g/mol)
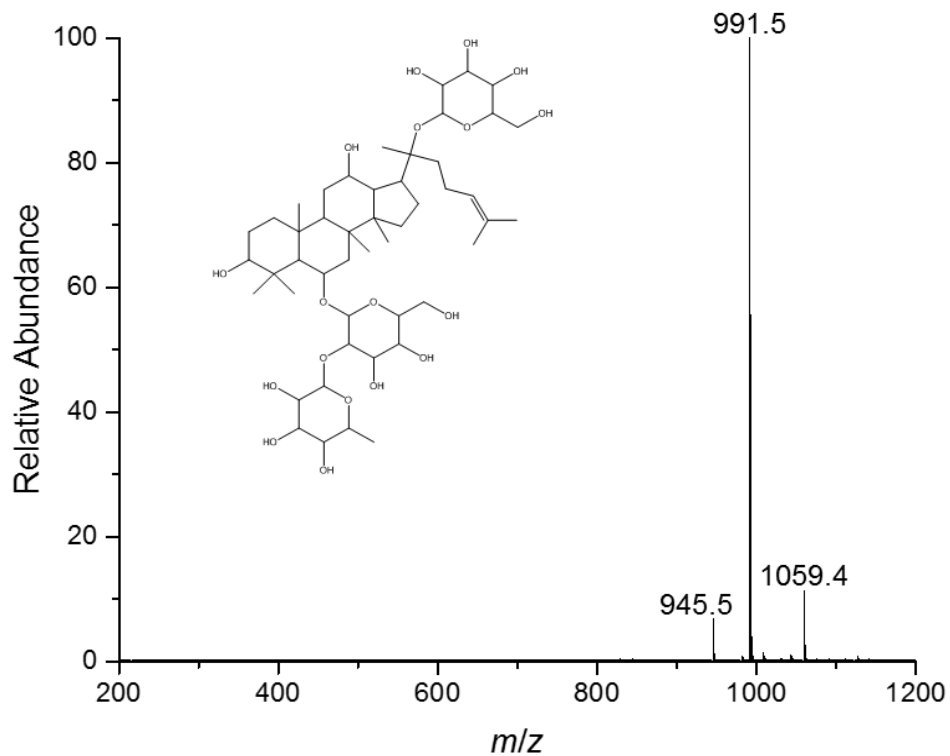
Figure B-6. Mass spectra and molecular structure of ginsenoside Re (947.17 g/mol)
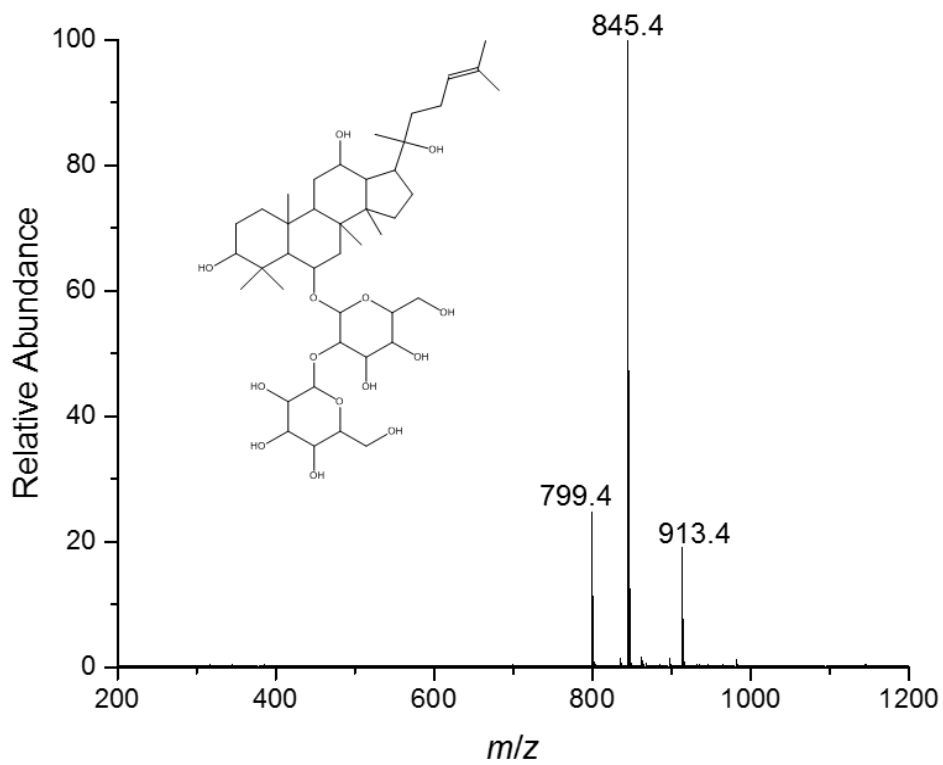
Figure B-7. Mass spectra and molecular structure of ginsenoside Rf (801.03 g/mol)
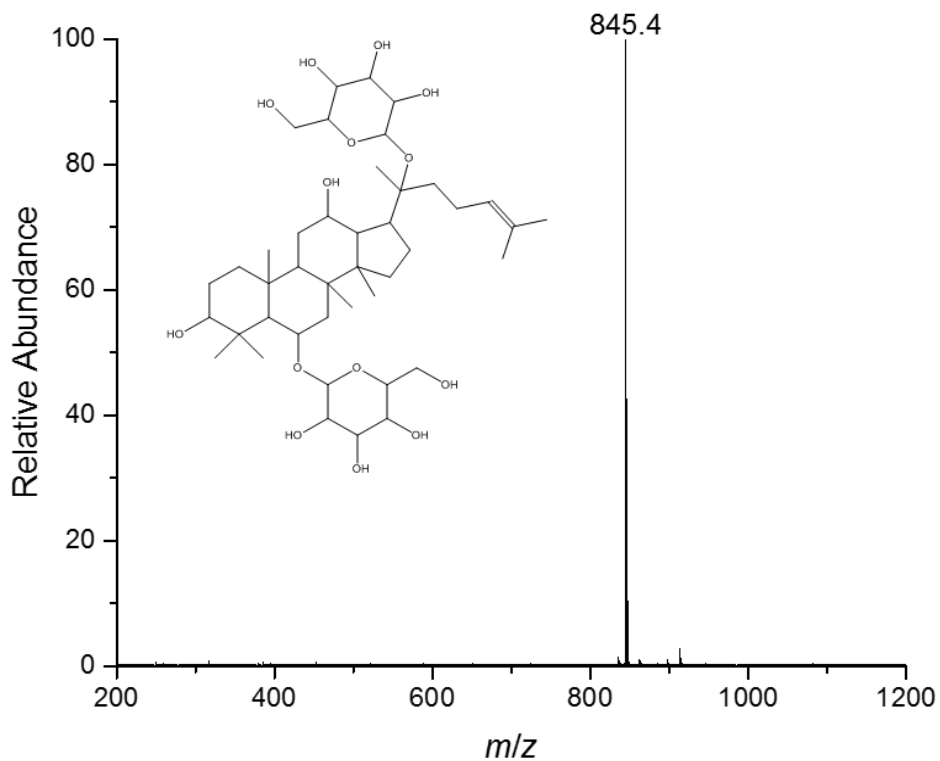
Figure B-8. Mass spectra and molecular structure of ginsenoside Rg1 (801.03 g/mol)
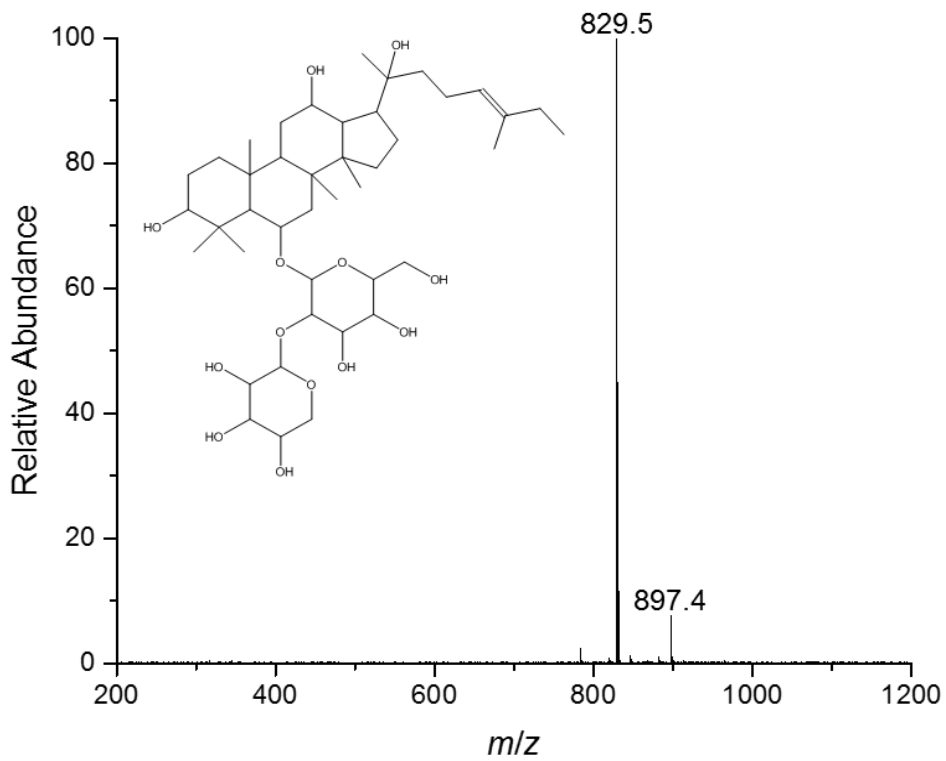
Figure B-9. Mass spectra and molecular structure of ginsenoside Rg2 (785.03 g/mol)

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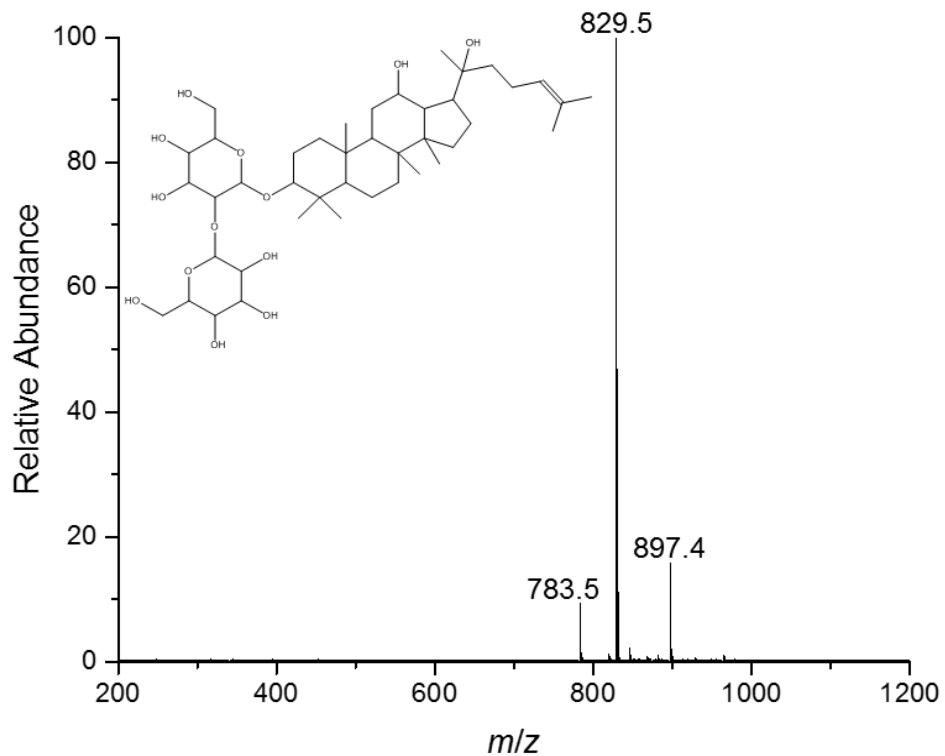
Figure B-10. Mass spectra and molecular structure of ginsenoside Rg3 (785.03 g/mol)
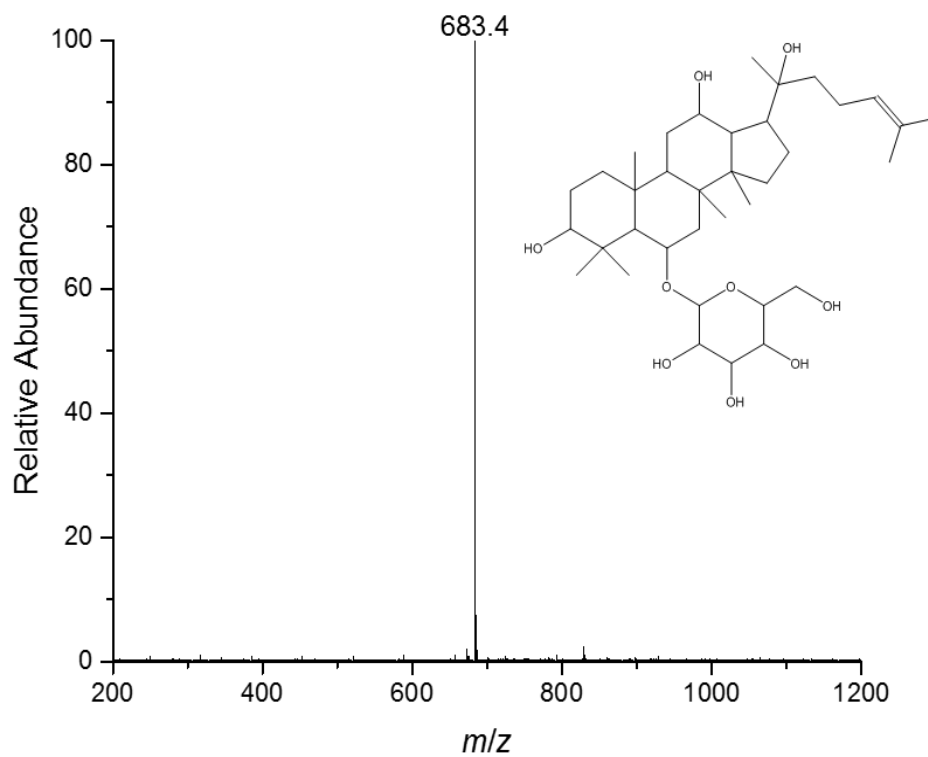
Figure B-11. Mass spectra and molecular structure of ginsenoside Rh1 (638.89 g/mol)

19
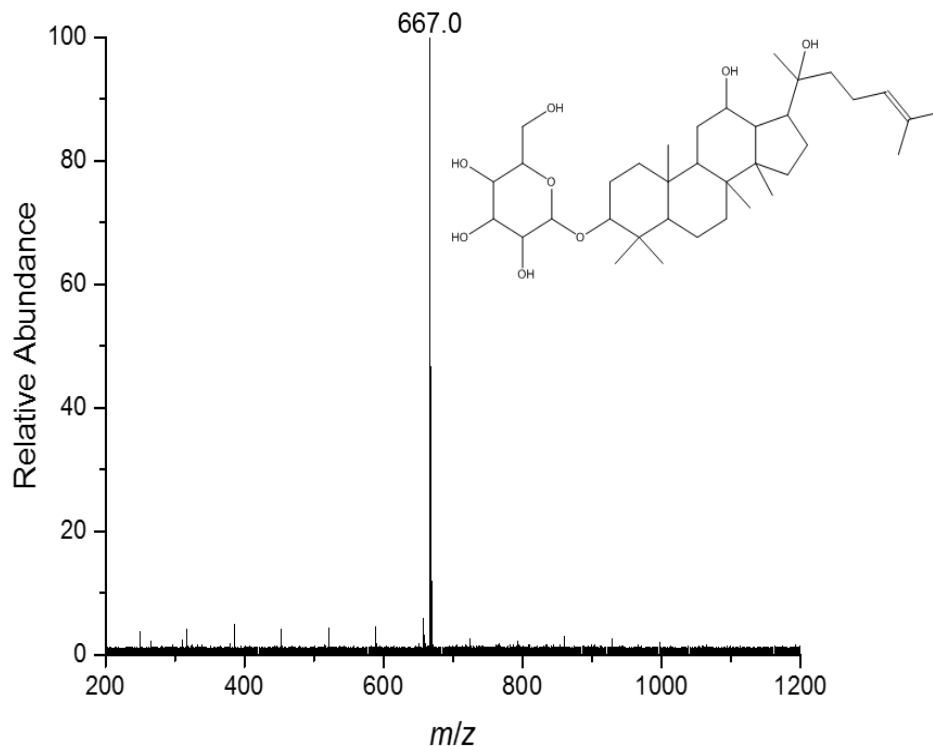
Figure B-12. Mass spectra and molecular structure of ginsenoside Rh2 (622.89 g/mol)

LC-MS Analysis of Ginsenoside Mixtures
The goal of this part of the project was to develop a new LC-MS method for separating all the ginsenosides present in SRM 3389. Based on LC-UV method development discussed in Appendix A, an ACE 3 C_{18} (150 mm × 4.6 mm) with a 3 µm particle size was selected for all LC-MS studies. The optimized separation conditions from the initial study included a 0.7 mL/min flowrate, 25 °C column temperature, absorbance wavelength of 200 nm, and the mobile phase gradient summarized in Table B-2. These conditions allowed for the baseline separation of all the ginsenoside components present in the CRM MIGS-1 and SRM 3389 solutions, a requirement for the SRM 3389 certification measurements.

Table B-2. Mobile phase gradient for the LC-MS method

Time	H$_2$O/0.1% FA (%)	ACN (%)
0.0	78	22
10.0	78	22
38.0	42	58
45.0	0	100
60.0	0	100
65.0	78	22
70.0	78	22

The LC-MS chromatograms in selective ion monitoring (SIM) mode for the 12 ginsenosides using the previously optimized LC conditions are shown in Figure B-13. Under these conditions, Rb2 and Rb3 partially co-elute within respect to the *m/z* 1123.5 ion chromatogram. The 10 remaining

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ginsenosides are baseline resolved in their respective ion chromatograms. Rb2 and Rb3 have been identified previously in the SRM 3389, Cerilliant calibration solutions and CRM MIGS-1 from NRC Canada. The baseline separation of these two ginsenosides is critical to the certification measurements of SRM 3389. For this reason, the separation conditions for the LC-MS measurements were slightly adjusted to achieve a baseline separation for Rb2 and Rb3. The LC-MS chromatograms in the SIM mode obtained with the new separation conditions are shown in Figure B-14.
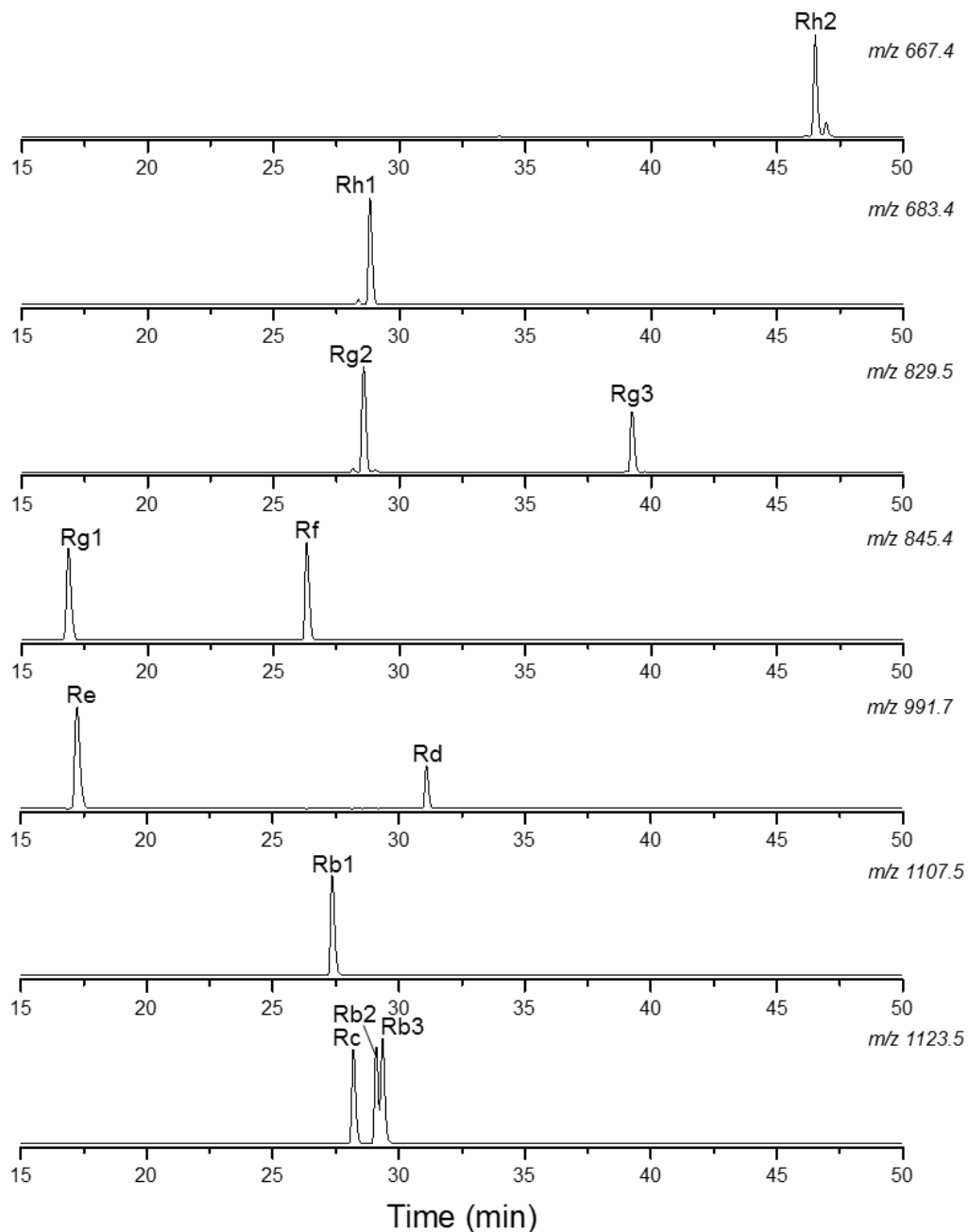
Figure B-13. LC-MS chromatograms in SIM mode under initial separation conditions

The separation conditions included a 0.7 mL/min flowrate, 25 °C column temperature, and the mobile phase gradient summarized in Table A-2.

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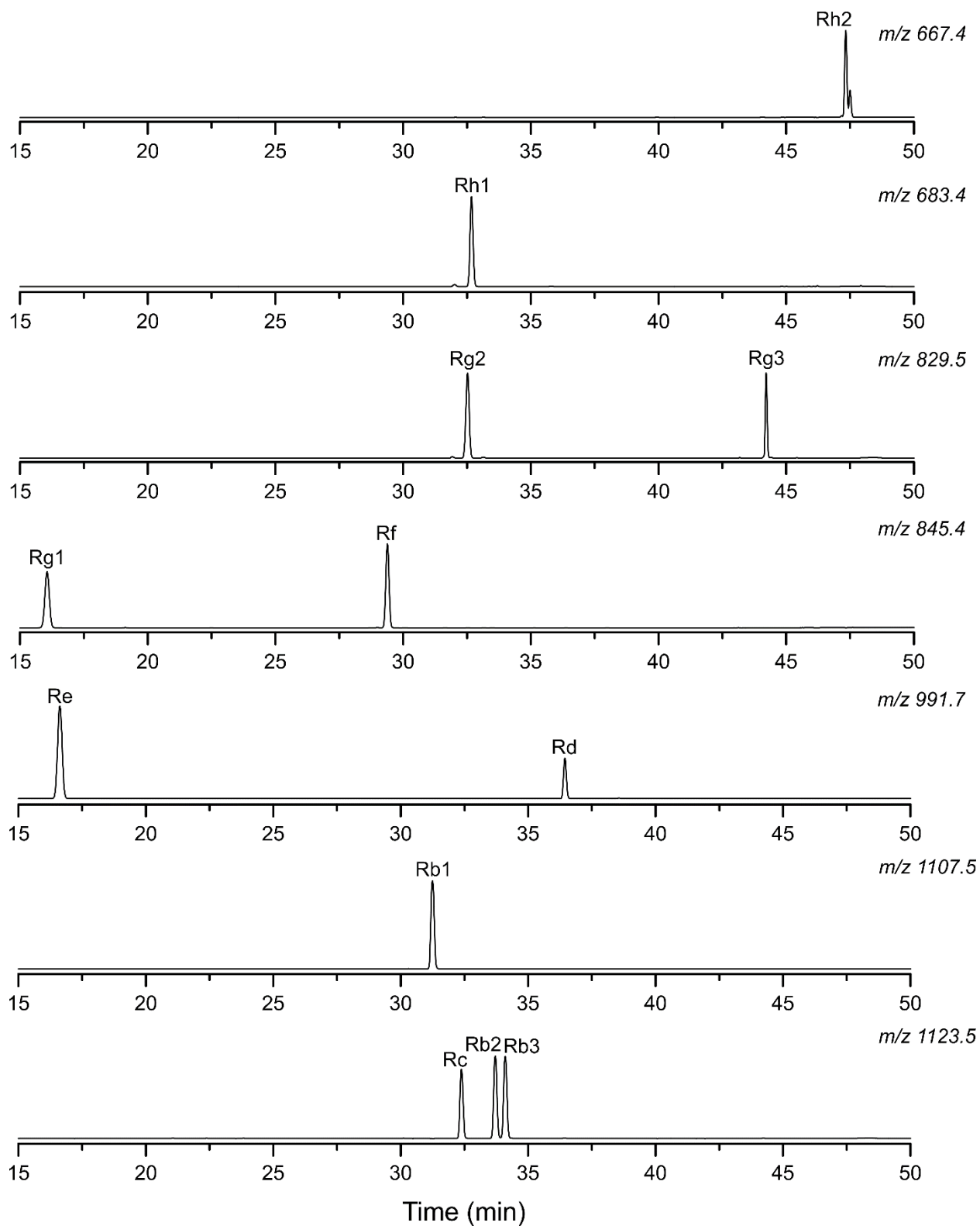
Figure B-14. LC-MS chromatograms in SIM mode under optimized separation conditions

The separation conditions included a 0.7 mL/min flowrate, 25 °C column temperature, and the mobile phase gradient summarized in Table B-2.

B. Conclusions

An LC-MS method was developed to separate a mixture of 12 ginsenosides, which was used in future certification measurements to determine the mass fractions of SRM 3389 ginsenoside calibration solution. The flowrate, initial mobile phase composition, and column temperature were chosen based on previous LC-UV studies. However, the mobile phase gradients required some modification to allow for the baseline separation of ginsenoside Rb2 and Rb3. Mass spectra were collected for all the ginsenosides in negative ion mode, which allowed for the appropriate choice of m/z ions to be determined and provided additional chemical identity. The FA adduct ion, $[M - 2H + HCO_2H]^-$, provided the highest signal abundance in most of the mass spectra.

B. References

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[B3] J. Liu, Y. Liu, L. Zhao, Z.H. Zhang, Z.H. Tang, Profiling of ginsenosides in the two medicinal Panax herbs based on ultra-performance liquid chromatography-electrospray ionization-mass spectrometry, SpringerPlus, 5 (2016) 11.

[B4] X. Miao, C.D. Metcalfe, C. Hao, R.E. March, Electrospray ionization mass spectrometry of ginsenosides, Journal of Mass Spectrometry, 37 (2002) 495-506.

Appendix C

Certification of SRM 3389 Ginsenoside Calibration Solution by LC-UV and LC-MS

C. Materials

The reference standards for ginsenoside Rb3, Rf, Rg2, Rg3, Rh1, and Rh2 were obtained from Cerilliant. The purities of these standards were previously assigned by PhytoLab by HPLC and are listed in Table A-1. The reference standards for ginsenoside Rb1, Rb2, Rc, Rd, Re, and Rg1 were obtained from NRC of Canada. The purities of these standards are listed in Table C-1 and were assigned at INMS of NRC Canada in 2011 by q^{1}H-NMR using external standards and NIST SRM 350b benzoic acid. CRM MIGS-1 (Ginsenoside Mixture) was obtained from the NRC of Canada. SRM 3389 preparations are described detail previously on page 9. HPLC grade ACN and H_2O was purchased from Fisher Scientific. The COAs relevant to the metrological traceability of SRM 3389 are reproduced in Appendix E.

Table C-1. Purity information on the NRC Canada ginsenoside reference standards

Ginsenoside	Batch #	Purity[a] (%)	u[b] (%)	U_{95}[c] (%)	TGA moisture[d] mg/g (±1s)
Rb1	AW110725Rb1	96.7	0.05	0.10	19.0 (0.3)
Rb2	AW110725Rb2	88.8[e]	0.05	0.10	17.9 (3.1)
Rc	AW110725Rc	92.5	0.06	0.12	61.9 (1.7)
Rd	AW110725Rd	89.7	0.07	0.14	75.3 (1.0)
Re	AW110725Re	97.8	0.05	0.10	13.7 (1.0)
Rg1	AW110725Rg1	96.5	0.6	0.12	17.3 (0.4)

[a] Purities determined by external standard q^{1}H-NMR using NIST SRM 350b benzoic acid, $n = 5$ except Rb2 and Rc where $n = 3$. q^{1}H-NMR percentages are on a wet weight basis.
[b] Standard uncertainty.
[c] Expanded uncertainty.
[d] Based on triplicate measurement determined from loss on drying at 110 °C.
[e] Rb3 was identified as an impurity in the Rb2 standard with a mass fraction by q^{1}H-NMR of 85 mg/g, $u = 10$ mg/g.

C. Sample Preparation

For the preliminary studies, small portions of each compound listed in Table A-1 were dissolved in 1 mL aliquots of methanol. A 12-component ginsenoside mixture was prepared by combining small quantities (< 100 µL) of each ginsenoside solution. For the certification measurements, three calibration solutions (calibrant 1, 2, and 3) were prepared using the reference standards listed in Table C-1. The masses used to prepare calibrants 1, 2, and 3 are listed in Table C-2.

Table C-2. Calibrant masses and mass fractions for the certification measurements

	Calibrant 1			Calibrant 2			Calibrant 3		
			Corrected[a]			Corrected[a]			Corrected[a]
		Mass	Mass		Mass	Mass		Mass	Mass
	Mass	Fraction	Fraction	Mass	Fraction	Fraction	Mass	Fraction	Fraction
Ginsenoside	(mg)	(mg/g)	(mg/g)	(mg)	(mg/g)	(mg/g)	(mg)	(mg/g)	(mg/g)
Rg1	1.493	0.309	0.298	1.979	0.411	0.396	1.349	0.281	0.272
Re	5.772	1.194	1.170	1.273	0.264	0.258	2.050	0.428	0.418
Rb1	14.87	3.077	2.975	2.723	0.565	0.546	2.643	0.551	0.533
Rc	2.272	0.470	0.435	1.696	0.352	0.326	1.284	0.268	0.248
Rb2	2.760	0.571	0.507	1.798	0.374	0.331	2.188	0.456	0.405
Rd	1.126	0.233	0.209	1.016	0.211	0.189	0.928	0.194	0.174
Rf	4.106	0.850	0.799	2.087	0.433	0.407	3.124	0.651	0.612
Methanol	4833.41			4818.74			4794.76		

[a] Mass fraction values are corrected using the purity listed in Table C-1.

For the LC-UV certification measurements, one ampule of the CRM MIGS-1, two ampules of the single component SRM 3389, and five ampules of the six-component SRM 3389 were removed from the freezer to reach room temperature and the solutions were transferred to an LC autosampler vial for analysis. Only two ampules of the single component SRM 3389 was used because of a limited number in comparison to the six-component material. The five ampules of the SRM 3389 were randomly selected from containers across the production lot. 1 mL samples of the calibrant solutions were transferred to individual LC autosampler vials for analysis. The three calibrant solutions and CRM MIGS-1 control solution was measured a total of 10 times. The two ampules of the single component SRM 3389 was measured five times. The five ampules of the six component SRM 3389 was measured two times.

For the LC-MS certification measurements, the three calibrants, CRM MIGS-1, and SRM 3389 solutions were mixed with an internal standard solution of Rh1. Rh1 was selected as the internal standard because it is structurally related, chromatographically resolved, and not included in the SRM solution. 1.602 mg of Rh1 was weighed out and dissolved in 6442.65 mg of methanol. The internal standard was stored in a screw cap amber vial. Small quantities of the internal standard (Rh1) solution were weighed out and mixed with masses of the three calibrant solutions, CRM MIGS-1 solution, and the SRM 3389 solutions (Table C-3). These samples were measured the same number of times as the LC-UV measurements.

Table C-3. Sample and internal standard solution masses for certification by LC-MS.

Sample	Sample Solution (mg)	Internal Standard (mg)
Cal 1	0.15831	0.16961
Cal 2	0.16113	0.16278
Cal 3	0.16290	0.16208
Control	0.16293	0.16182
SRM Rf 1_1	0.11839	0.11775
SRM Rf 1_2	0.11960	0.12012
SRM 6_1	0.11886	0.12106
SRM 6_2	0.11890	0.12003
SRM 6_3	0.12032	0.11902
SRM 6_4	0.07930	0.07806
SRM 6_5	0.05655	0.07790

C. Instrumental Methods

LC-UV Method.
The LC-UV measurements were performed using the same instrumentation and optimized separation conditions described in Appendix A.

LC-MS Method.
The LC-MS measurements were performed using the same instrumentation and optimized separation conditions described in Appendix B.

C. Results and Discussion

The goal of this part of the project was to perform the certification measurements for a SRM 3389 Ginsenoside Calibration Solution. The calibration solution consists of two different ampule solutions: (1) six component mixture of ginsenoside Rb1, Rb2, Rc, Rd, Re, and Rg1 and (2) single component ginsenoside Rf. Preliminary stability of the six-component SRM 3389 was analyzed four times. The resultant peak areas ratios are reported in Table C-4 Table 1with the peak areas and ratios from previous LC-UV analysis in 2013. By comparison of these values, there is not a significant difference in peak area ratios of Rb1, Rc, Rb2, and Rd. The ratio of Rg1 is slightly higher in the present study (0.392) than in 2013 (0.379), although the uncertainty is not reported for the 2013 LC-UV measurement. In addition, the two analyzes were performed on different instruments with different detectors, which may account for the slight differences in the ratios.

Table C-4. Peak area ratios for five ginsenosides in SRM 3389

Ginsenoside	2013 ($n = 1$)	2015 ($n = 4$)
Rg1	0.379	0.392 ± 0.003
Rb1	2.130	2.123 ± 0.001
Rc	0.446	0.444 ± 0.001
Rb2	0.506	0.505 ± 0.001
Rd	0.170	0.170 ± 0.001

The schematic diagram for the certification plan is shown in Figure C-1. Based on previous methods, the certification measurements utilized an LC-UV (Appendix A) and LC-MS (Appendix B) methods, that have the capacity to resolve a 12 ginsenoside mixture. A summary of the certification measurements is reported in Table C-5 and these results are purity corrected. The mass fraction values reported for both LC methods are similar and to the initial gravimetry values from the preparation of the calibration solutions. The CRM MIGS-1 calibration solution from NRC Canada was used as the control for the measurements. The LC-UV and LC-MS methods provided comparable mass fraction values to the reported values in the CRM MIGS-1 COA. The following sections discuss the certification measurements in more detail.

SRM Preparation

Analysis #1

Analysis #2

**Pure Solids
(NRC lot 2008)**
6 different compounds
Purity measured 2008 NRC

**Pure Solids
(NRC lot AW110725)**
6 different compounds
Purity measured 2011 NRC

(Phytolab)
Compound Rf

**Pure Solids
(NRC lot AW110725)**
6 different compounds
Purity measured 2011 NRC

(Phytolab)
Compound Rf

**solution
preparation
Cerilliant**

**Control
MIGS-1**

**Control
MIGS-1**

**Candidate
SRM 3389**

gravimetry

**Candidate
SRM 3389**

LC/UV

**Candidate
SRM 3389**

LC/MS

confirmatory

SRM 3389

certified values

Figure C-1. Schematic diagram for the certification of SRM 3389

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Table C-5. Summary of results for SRM 3389 and CRM MIGS-1 calibration solutions

Ginsenoside[a]	Gravimetry[c] Mass Fraction (mg/g)	LC-UV[d] Mass Fraction[e] (mg/g)	RSD (%)	LC-MS[f] Mass Fraction[e] (mg/g)	RSD (%)
SRM 3389					
Rg1	0.51	0.518 ± 0.013	2.53	0.530 ± 0.023	4.27
Re	1.53	1.55 ± 0.039	2.52	1.54 ± 0.095	6.13
Rf[b]	1.27	1.31 ± 0.039	3.02	1.31 ± 0.138	10.59
Rb1	3.81	3.80 ± 0.097	2.55	3.86 ± 0.345	8.92
Rc	0.76	0.761 ± 0.019	2.55	0.750 ± 0.059	7.81
Rb2	0.89	0.903 ± 0.023	2.54	0.877 ± 0.078	8.85
Rd	0.25	0.251 ± 0.008	3.18	0.246 ± 0.025	8.68
MIGS-1: Control					
Rg1	0.258 ± 0.005	0.254 ± 0.005	1.90	0.257 ± 0.015	5.67
Re	0.506 ± 0.002	0.504 ± 0.009	1.86	0.525 ± 0.028	5.40
Rf	-	-	-	-	-
Rb1	1.249 ± 0.016	1.24 ± 0.024	1.96	1.35 ± 0.114	8.42
Rc	0.505 ± 0.005	0.489 ± 0.010	1.98	0.497 ± 0.044	8.89
Rb2	0.252 ± 0.002	0.254 ± 0.005	2.02	0.261 ± 0.017	6.39
Rd	0.507 ± 0.006	0.489 ± 0.010	1.91	0.504 ± 0.055	10.92

[a] Ginsenosides are listed in order of LC elution on the ACE 3 C_{18} column.
[b] The mass fraction value for Rf will be in the certificate of analysis as a non-certified value.
[c] The gravimetry mass fraction values are estimates and were supplied to NIST from Cerilliant.
[d] LC-UV values quantified using an external calibration approach.
[e] The mass fraction values are the average ± standard deviation of ten measurements for each calibrant, SRM 3389, and CRM MIGS-1 control solutions. In the cases of LC-MS, the SRM 3389 was measured nine times.
[f] LC-MS values quantified using ginsenoside Rh1 for the internal standard calibration approach.

LC-UV Certification Measurements.

The LC-UV quantitation measurements were based on external standard calibration method. External standard calibration method was suitable for the LC-UV measurements because no manipulation of the SRM 3389 or CRM MIGS-1 solutions was required. The external standard calibration method in the present study used the linear regression relationship between the chromatographic peak area and the mass fraction (mg/g) of the ginsenoside standards in three calibrants in the ginsenoside solutions. Each calibrant was measured ten times and the calibration curves are shown in Figure C-2. In all plots, the correlation coefficients (R^2) were greater than or equal to 0.9989 demonstrating excellent linearity for the external standard calibration method.
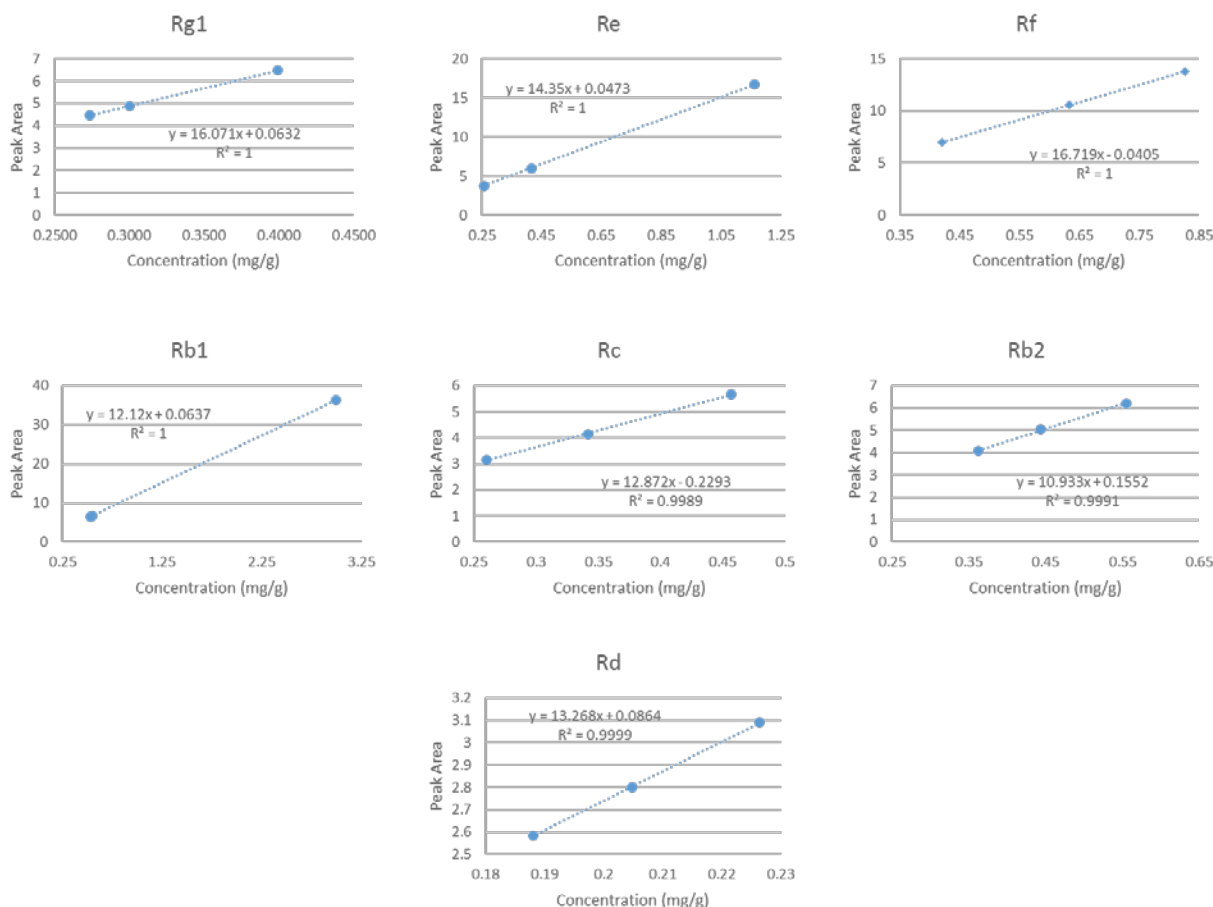
Figure C-2. Calibration curves for the LC-UV external calibration of SRM 3389

The main concern with external standard calibration measurements is the reproducibility of the injection volume. To minimize the injection volume error, the autosampler injection syringe was purged to remove air and the performance of the autosampler was verified prior to starting certification. Acetone was the solvent chosen to test the autosampler because of its short retention time (≈ 1.20 min) and strong absorbance signal at 200 nm. Multiple injections of acetone were made under isocratic conditions of 100 % ACN and a flowrate of 1.5 mL/min. The first 10 injections were ignored to allow for the system to reach equilibrium. The peak areas from the remaining injections are plotted in Figure C-3. The average peak area of the acetone measurements was 85.97 with a relative standard deviation (RSD) of 0.48 % ($n = 26$).

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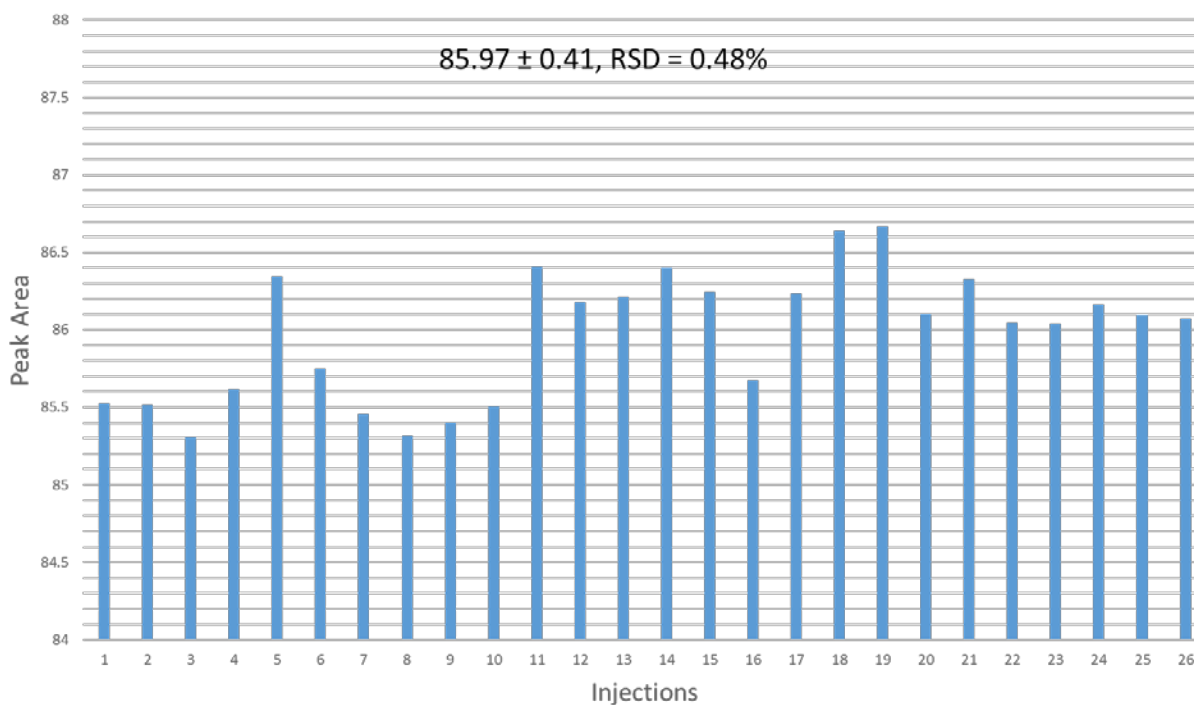
Figure C-3. Injection reproducibility for the LC-UV method using acetone

The LC-UV chromatograms of the three calibrant solutions, CRM MIGS-1 solution (control), and the SRM 3389 solutions are shown in Figure C-4. All the ginsenosides in the solutions are baseline resolved in the LC chromatograms and provide a signal ≈ 30 times the noise. A summary of the determined mass fraction values for the LC-UV method is reported in Table C-5 and have been corrected for the purity listed in Table C-1. The individual mass fraction values for each LC-UV measurement is shown in Table C-6 and Table C-7. In the case of SRM 3389, the mass fraction values are in good agreement with the values provided from the gravimetry and LC-MS method. In the case of the CRM MIGS-1, the mass fraction values are in good agreement with the values reported in the COA (Appendix D) and LC-MS method. Ginsenoside Rf was not present in CRM MIGS-1. The LC-UV measurements ($n = 10$) provide good precision for the SRM 3389 and CRM MIGS-1 with RSD values of ≈ 3 % and ≈ 2 %, respectively. The peak area vs. run order plots in Figure C-5 show that there was no apparent trend in the measurements and supports that vial to vial homogeneity exists for SRM 3389.
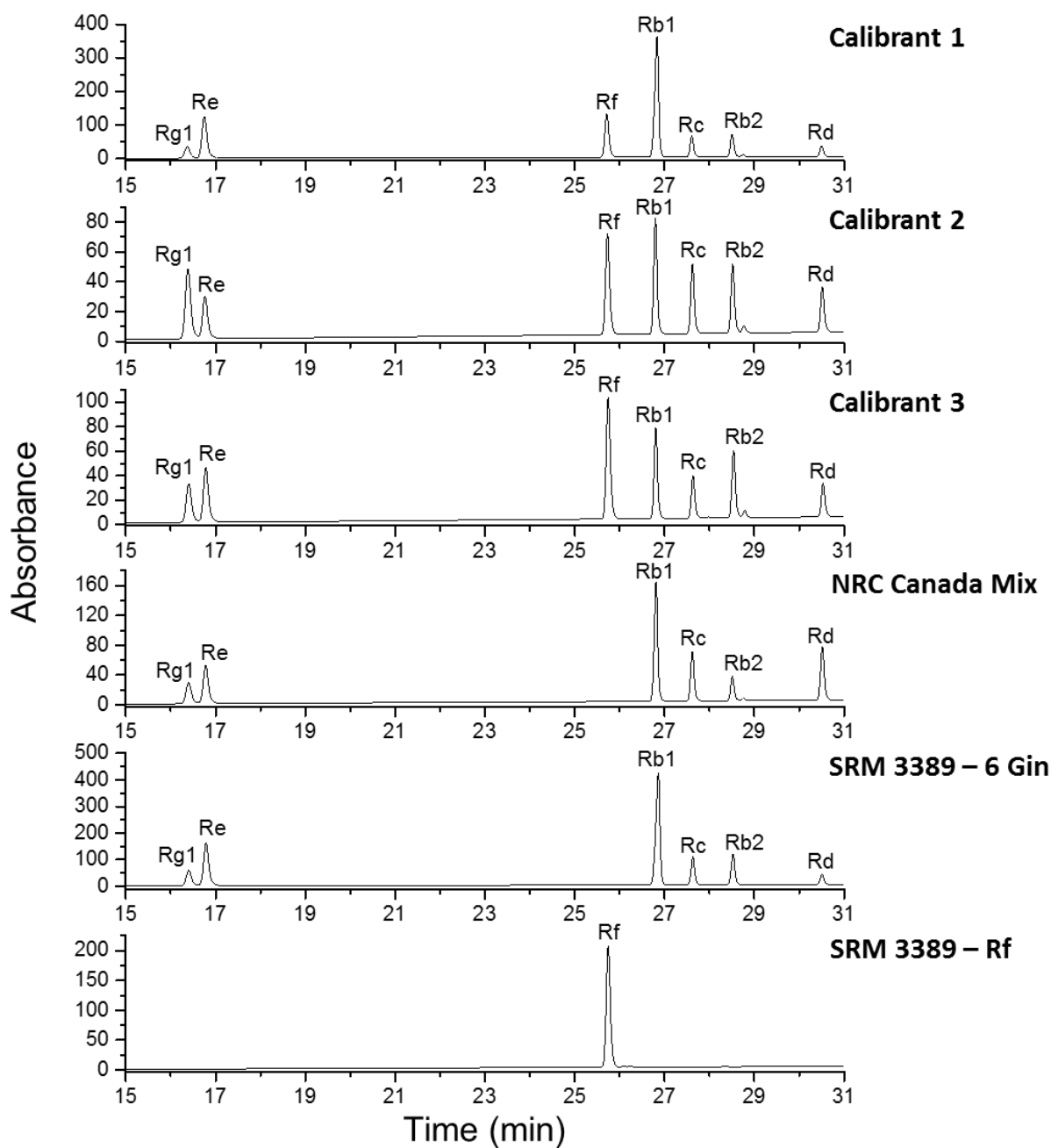
Figure C-4. LC-UV separations of the different certification solutions for SRM 3389

Table C-6. Purity-corrected mass fractions of SRM 3389 LC-UV measurements (mg/g)

	Rg1	Re	Rf	Rb1	Rc	Rb2	Rd
Measurement 1	0.517	1.55	1.29	3.80	0.759	0.900	0.250
Measurement 2	0.531	1.59	1.34	3.89	0.779	0.924	0.258
Measurement 3	0.537	1.61	1.33	3.93	0.782	0.931	0.262
Measurement 4	0.535	1.61	1.29	3.92	0.785	0.930	0.260
Measurement 5	0.506	1.52	1.26	3.70	0.739	0.878	0.242
Measurement 6	0.525	1.57	1.38	3.84	0.768	0.911	0.254
Measurement 7	0.506	1.52	1.33	3.71	0.741	0.880	0.245
Measurement 8	0.503	1.51	1.29	3.68	0.736	0.875	0.244
Measurement 9	0.518	1.55	1.29	3.78	0.756	0.898	0.249
Measurement 10	0.505	1.52	1.26	3.70	0.738	0.876	0.237
Average	0.518	1.55	1.31	3.80	0.761	0.903	0.251
SD	0.013	0.039	0.039	0.097	0.019	0.023	0.008
RSD (%)	2.53	2.52	3.02	2.55	2.55	2.54	3.18

Table C-7. Purity-corrected mass fraction of CRM MIGS-1 LC-UV measurements (mg/g)

	Rg1	Re	Rf	Rb1	Rc	Rb2	Rd
Measurement 1	0.250	0.496		1.22	0.482	0.250	0.481
Measurement 2	0.245	0.487		1.19	0.470	0.244	0.470
Measurement 3	0.256	0.509		1.26	0.496	0.258	0.496
Measurement 4	0.254	0.503		1.24	0.488	0.254	0.488
Measurement 5	0.261	0.518		1.28	0.502	0.262	0.502
Measurement 6	0.255	0.507		1.25	0.491	0.255	0.490
Measurement 7	0.252	0.499		1.23	0.486	0.253	0.486
Measurement 8	0.261	0.517		1.28	0.502	0.261	0.501
Measurement 9	0.254	0.507		1.24	0.491	0.255	0.491
Measurement 10	0.252	0.501		1.23	0.485	0.252	0.486
Average	0.254	0.504		1.24	0.489	0.254	0.489
SD	0.005	0.009		0.024	0.010	0.005	0.009
RSD (%)	1.90	1.86		1.96	1.98	2.02	1.92
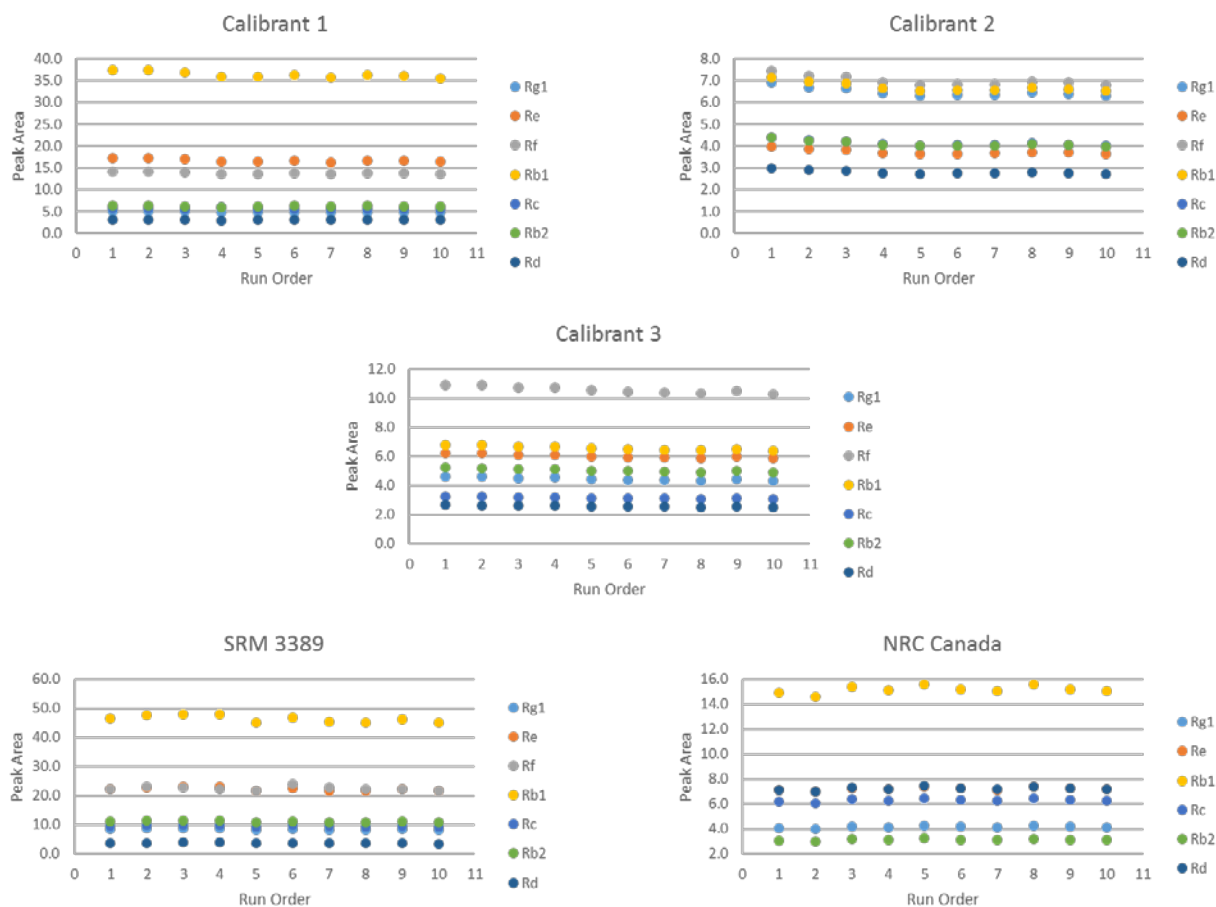
Figure C-5. LC-UV method reproducibility from run to run

LC-MS Certification Measurements.

The LC-MS quantitation measurements were based on the internal standard calibration method, typically us with MS. Internal standards allow for accurate quantitation measurements that are independent of injection reproducibility and detector response. The most important step is the choice of a suitable internal standard that meets the following criteria: (1) chemically similar, (2) similar detector response, (3) baseline resolved from all other ginsenosides of interest with similar retention times, and (4) stable. Isotopically labeled internal standards are preferred for quantitation; however, no isotopically labeled ginsenosides were commercially available at the time of analysis. For this reason, five ginsenosides (Rb3, Rg2, Rg3, Rh1, and Rh2) were investigated as possible internal standards for these quantitation measurements.

The LC-MS chromatograms in SIM mode for the seven ginsenosides present in SRM 3389 (Rg1, Re, Rf, Rb1, Rc, Rb2, and Rd) and the five-potential internal standard ginsenosides (Rg2, Rh1, Rb3, Rg3, and Rh2) are shown in Figure C-6. Rb3 could not be selected as the internal standard because it was found to be present as an impurity in the Rb2 reference standard. Rg3 and Rh2 elute significantly later in the chromatogram than all the ginsenosides present in the SRM 3389. Rg2 and Rh1 meet all the criteria listed above and either ginsenoside could have been selected as the internal standard. Rh1 was selected as the internal standard for certification measurements.

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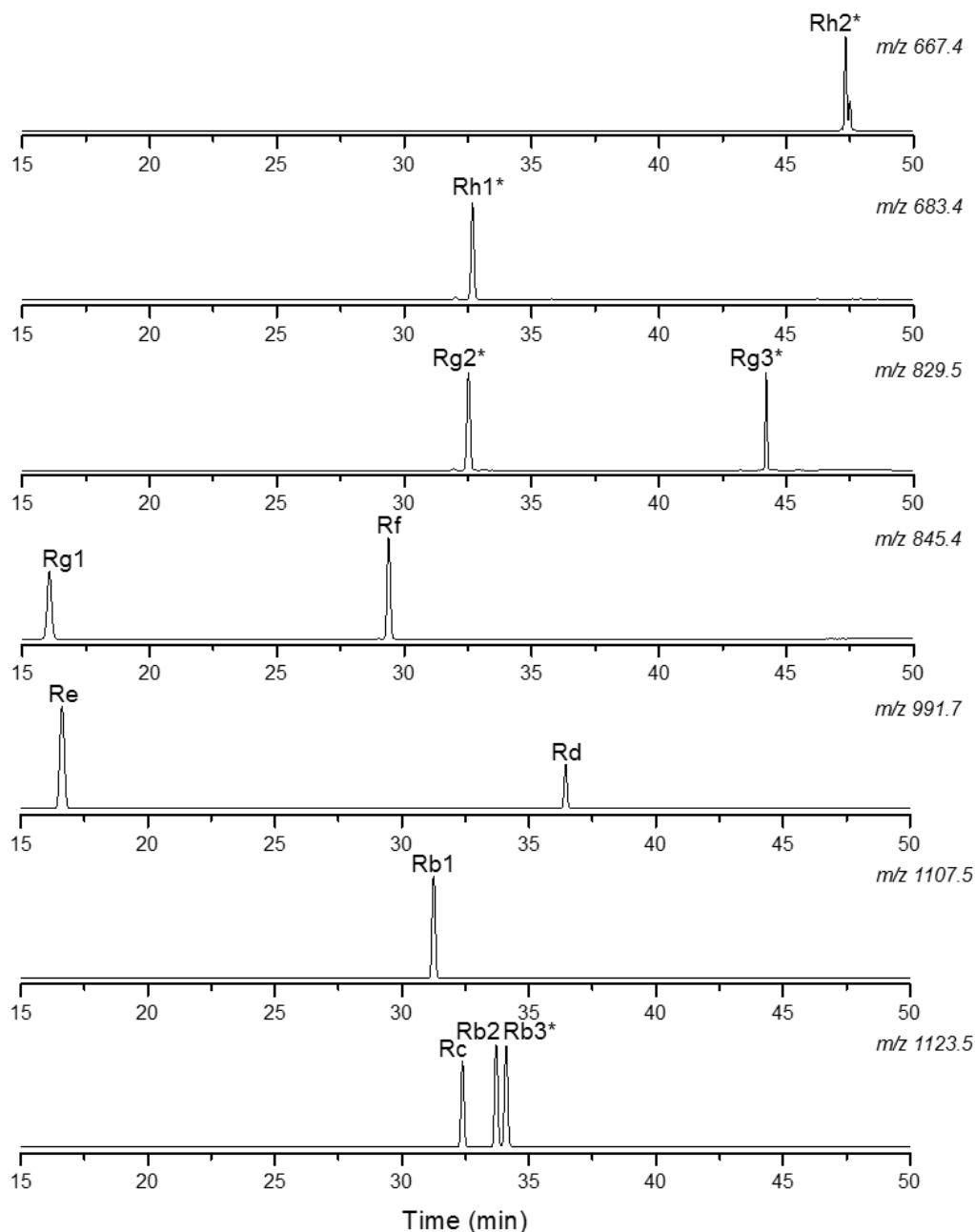
Figure C-6. LC-MS chromatograms in SIM mode under optimized separation conditions

The peak labels with an asterisk indicate possible internal standards.

The main difference from the external standard calibration method used in the LC-UV measurements was the sample preparation of the ginsenoside solutions. Small quantities of the calibrant solutions, MIGS-1 solution, and SRM 3389 were gravimetrical mixed with equivalent quantities of the internal standard in most cases. The three calibrants were measured ten times and the calibration curves are shown in Figure C-7. The R^2 values were greater than or equal to 0.9919 except for Rg1, which had an R^2 value of 0.8965.
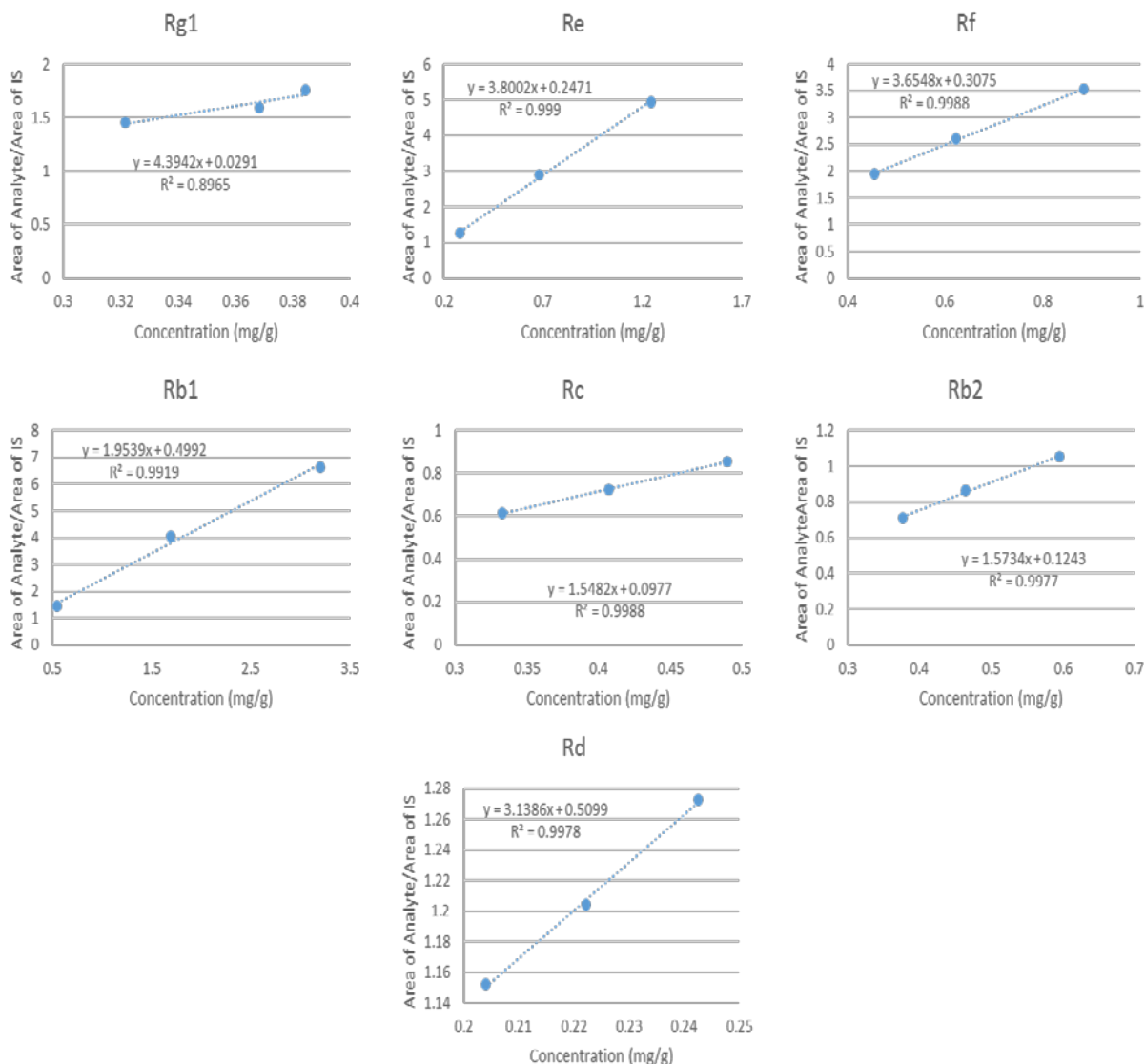
Figure C-7. Calibration curves for the internal standard calibration of SRM 3389

The LC-MS chromatograms in the selective ion monitoring (SIM) mode for calibrant 1, calibrant 2, calibrant 3, MIGS-1 solution (control), and the SRM 3389 solutions are shown in Figure C-8 to Figure C-12. All the ginsenosides in the solutions are baseline resolved and provide a signal intensity significantly greater than the noise. A summary of the determined mass fraction values for the LC-MS method is reported in Table C-8 and have been corrected for the purity listed in Table C-1. The individual mass fraction values for each LC-MS measurement is shown in Table C-8 and Table C-9. In the case of SRM 3389, the mass fraction values are in good agreement with the values provided from the gravimetry and LC-UV method. In the case of the MIGS-1, the mass fraction values are in good agreement with the values reported in the COA (Appendix E) and LC-UV measurements indicating that the measurements are in control. Considering the internal standard was not isotopically labeled, the LC-MS method provided good precision for the mass fraction values of SRM 3389 and CRM MIGS-1 with RSD values between 4.27 % (Rg1, SRM 3389) and 10.92 % (Rd, CRM MIGS-1).
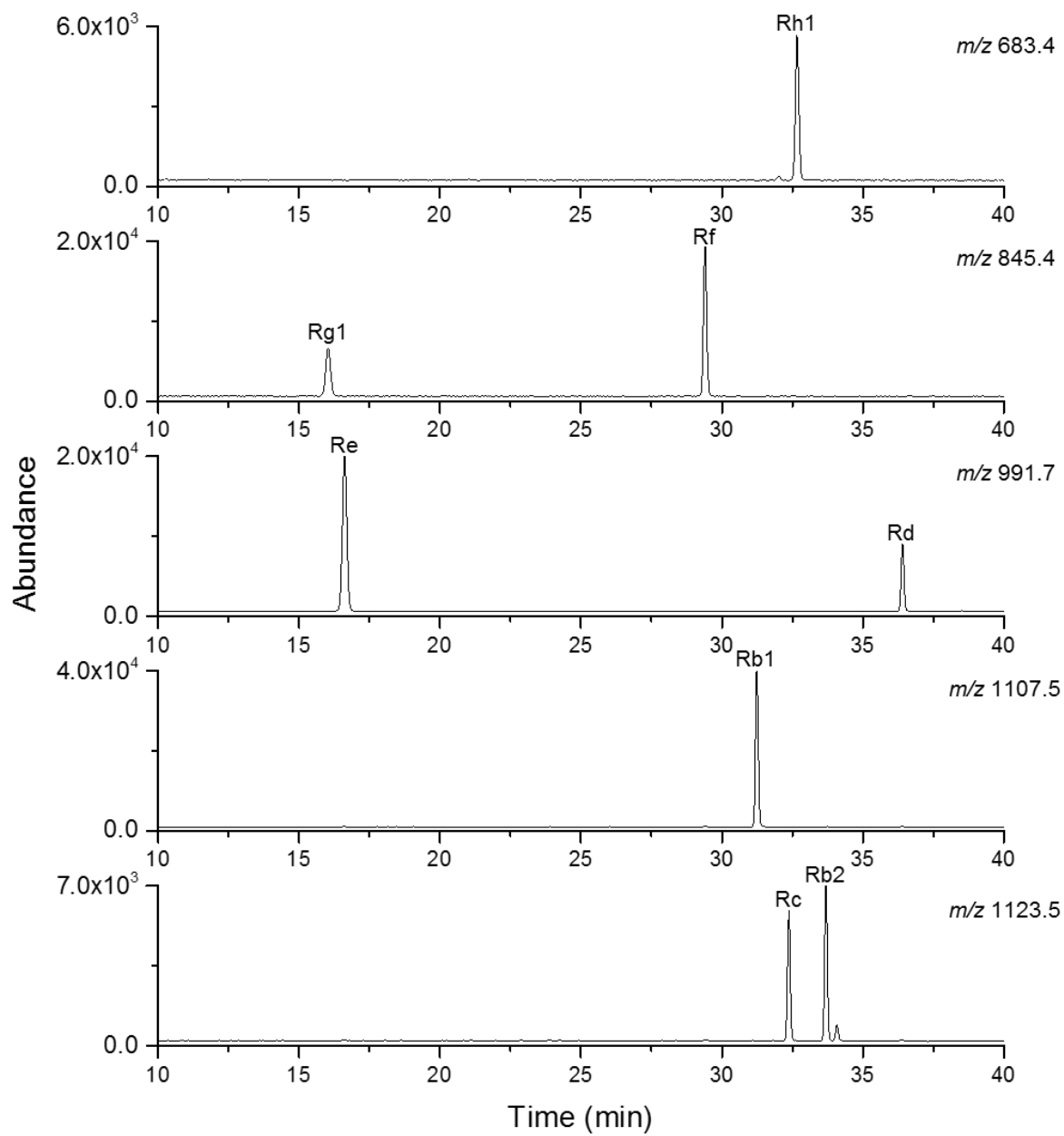
Figure C-8. LC-MS chromatograms in SIM mode for calibrant 1
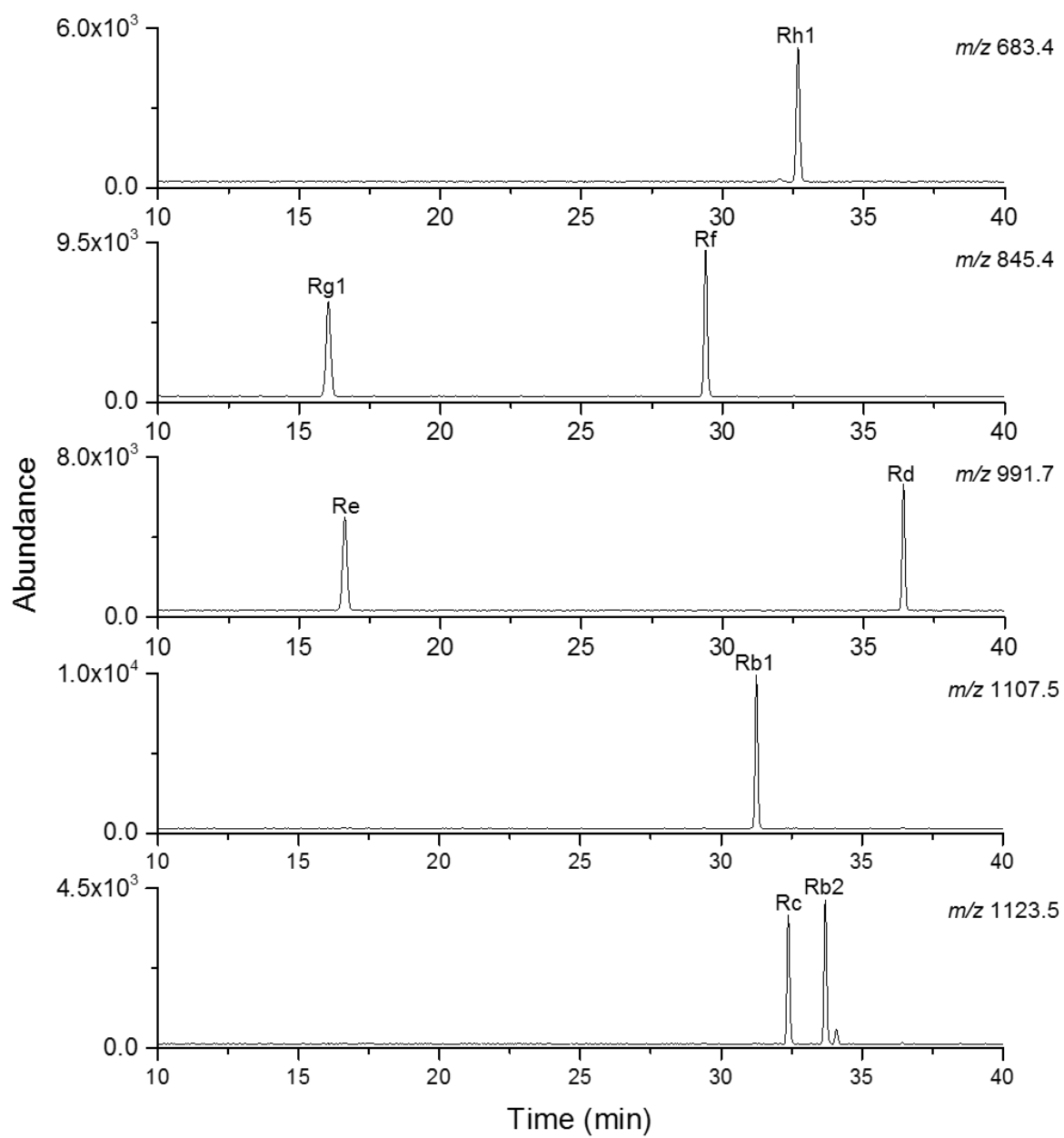
Figure C-9. LC-MS chromatograms in SIM mode for calibrant 2
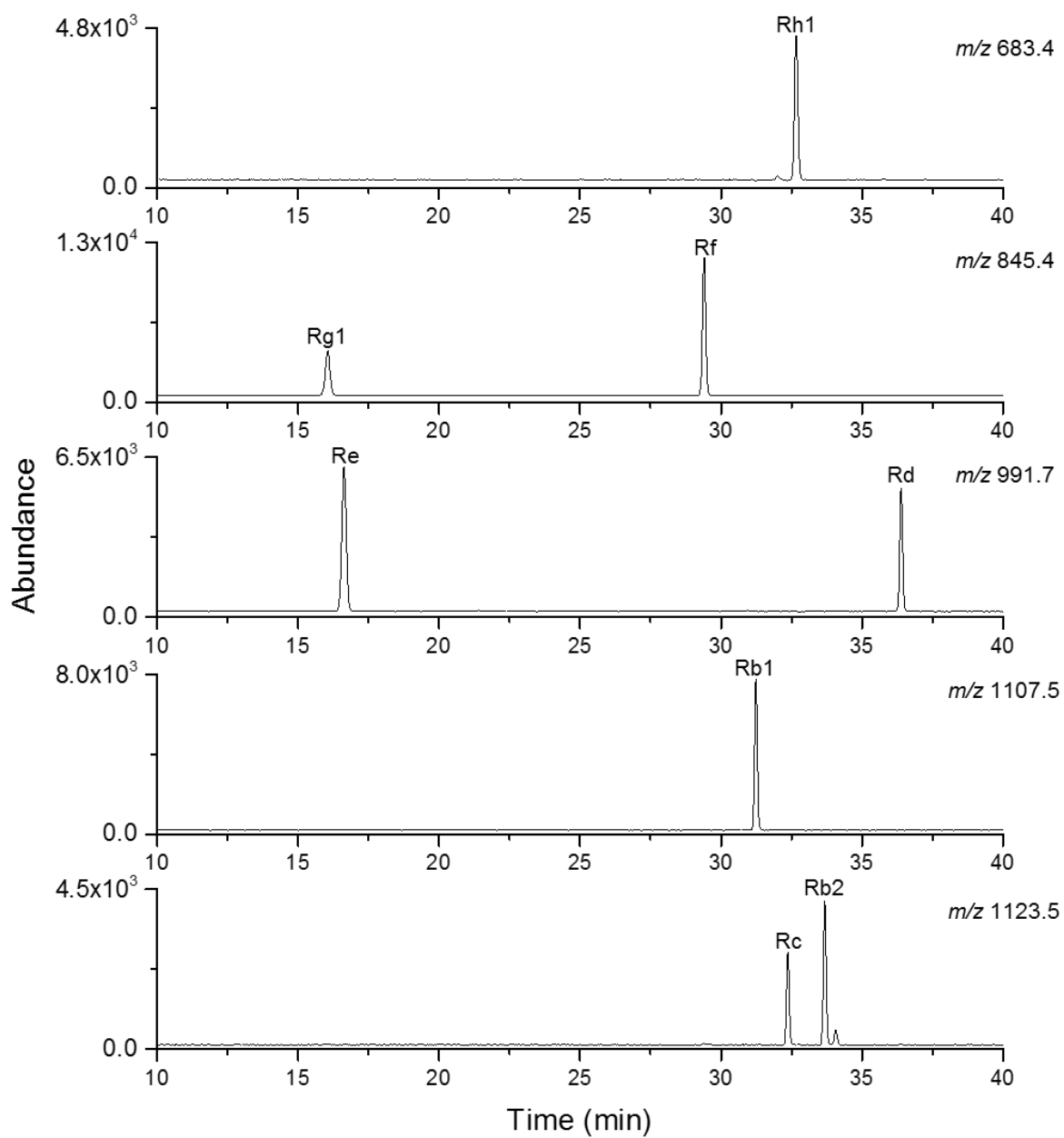
Figure C-10. LC-MS chromatograms in SIM mode for calibrant 3
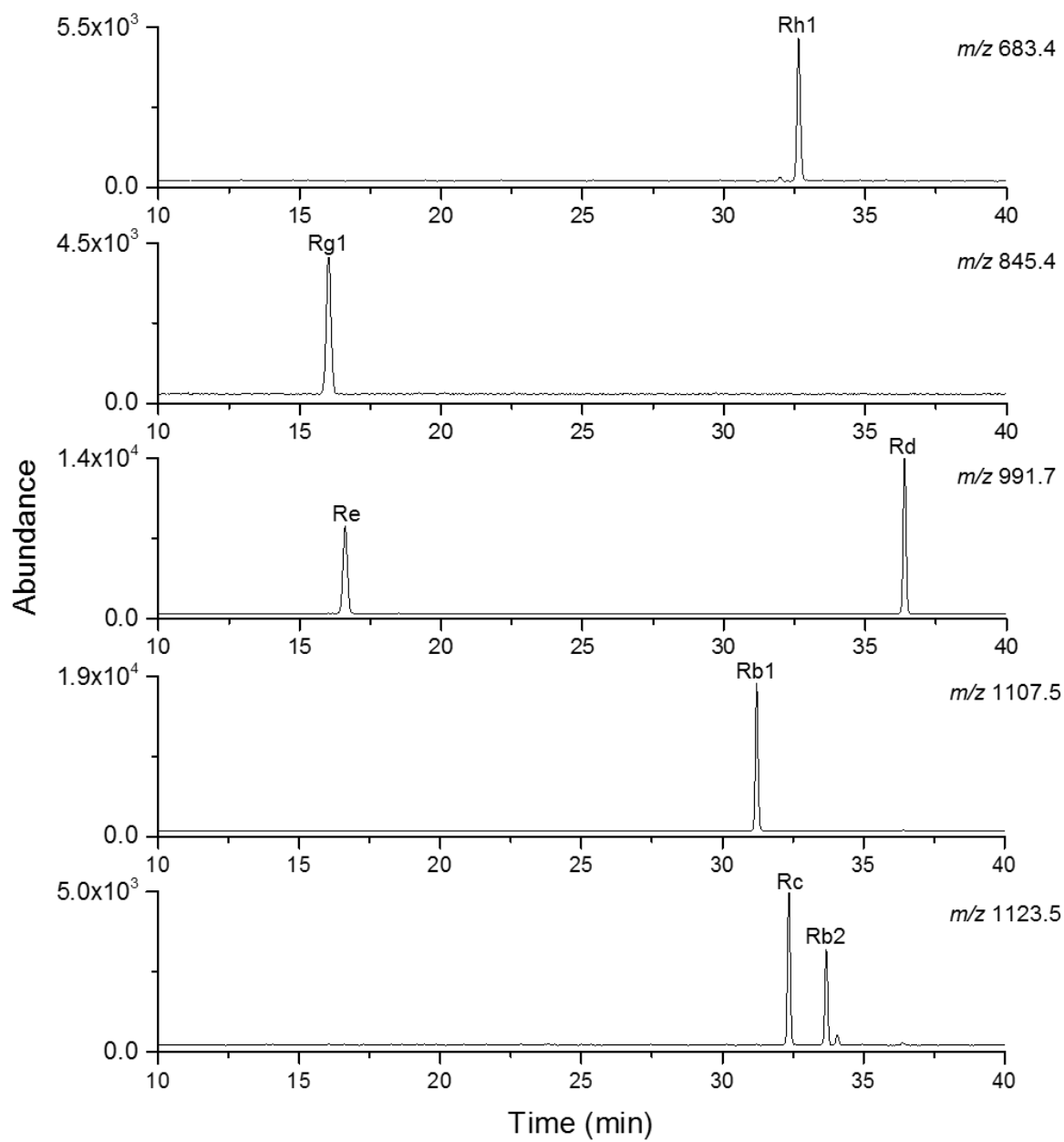
Figure C-11. LC-MS chromatograms in SIM mode for internal standard and CRM MIGS-1
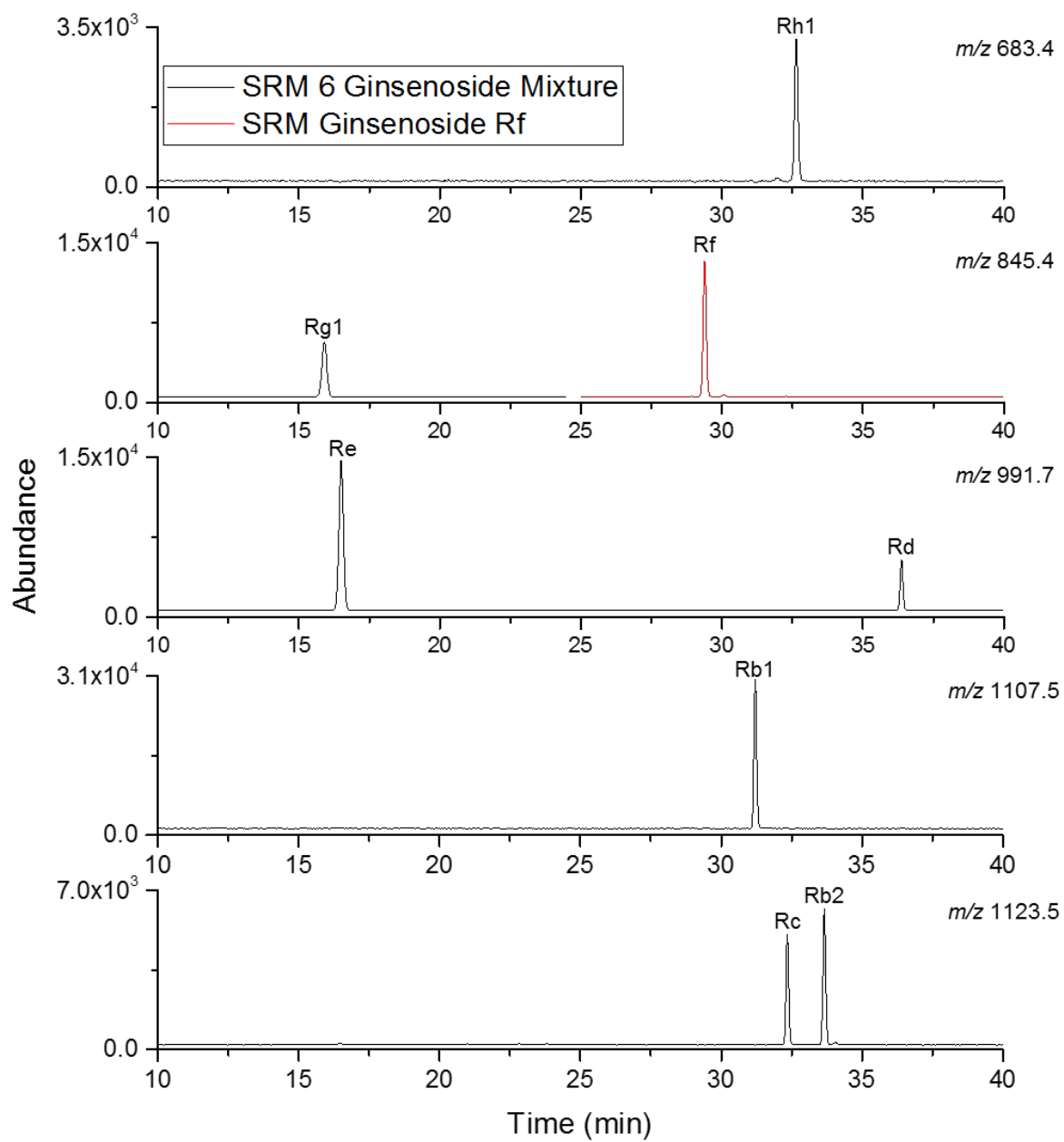
Figure C-12. LC-MS chromatograms in SIM mode for SRM 3389 and internal standard (Rh1)

Table C-8. Purity-corrected mass fractions of SRM 3389 LC-MS measurements (mg/g)

	Rg1	Re	Rf	Rb1	Rc	Rb2	Rd
Measurement 2	0.542	1.46	1.09	3.35	0.646	0.747	0.213
Measurement 3	0.495	1.39	1.19	3.41	0.689	0.772	0.213
Measurement 4	0.493	1.48	1.18	3.67	0.713	0.822	0.231
Measurement 5	0.552	1.66	1.24	3.69	0.801	0.931	0.255
Measurement 6	0.537	1.59	1.32	4.01	0.789	0.910	0.251
Measurement 7	0.549	1.61	1.40	4.27	0.803	0.957	0.269
Measurement 8	0.543	1.63	1.48	4.23	0.816	0.947	0.262
Measurement 9	0.515	1.53	1.47	4.01	0.736	0.892	0.257
Measurement 10	0.541	1.58	1.38	4.12	0.757	0.918	0.260
Average	0.530	1.54	1.31	3.86	0.750	0.877	0.246
SD	0.023	0.095	0.138	0.345	0.059	0.078	0.021
RSD (%)	4.27	6.13	10.59	8.93	7.81	8.85	8.68

Table C-9. Purity-corrected mass fraction of CRM MIGS-1 LC-MS measurements (mg/g)

	Rg1	Re	Rf	Rb1	Rc	Rb2	Rd
Measurement 1	0.279	0.561		1.21	0.442	0.245	0.426
Measurement 2	0.242	0.493		1.26	0.440	0.253	0.431
Measurement 3	0.234	0.500		1.29	0.462	0.261	0.461
Measurement 4	0.242	0.474		1.19	0.452	0.231	0.452
Measurement 5	0.254	0.526		1.35	0.517	0.256	0.526
Measurement 6	0.253	0.525		1.34	0.500	0.255	0.517
Measurement 7	0.267	0.539		1.45	0.537	0.287	0.550
Measurement 8	0.265	0.534		1.45	0.546	0.275	0.554
Measurement 9	0.270	0.560		1.51	0.524	0.276	0.555
Measurement 10	0.267	0.542		1.47	0.551	0.273	0.564
Average	0.257	0.525		1.35	0.497	0.261	0.504
SD	0.015	0.028		0.114	0.044	0.017	0.055
RSD (%)	5.67	5.40		8.42	8.90	6.39	10.9

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Appendix D

SRM 3389 Statistician's Report SRM 3389 Ginsenoside Calibration Solution

D. Assignment of values and uncertainties:

For each analyte, the certified or non-certified value is the mean of the method estimates available for that analyte. For an analyte, the method estimate is the mean of the measurements available for that analyte. The uncertainty of each method mean is the standard error of that mean. When the mass fraction value is based on more than one method, the uncertainty of the combined mean is estimated using a bootstrap procedure based on a Gaussian random effects model for the between-method effects [D1-D3]. The estimated values and their expanded uncertainties for the ginsenosides in SRM 3389 are listed in Table D-1.

Table D-1. SRM 3389 Ginsenoside Calibration Solution

Ginsenoside	Mass Fraction (mg/g)	U_k (mg/g)	k
Rg1	0.5240	0.0116	2.0
Re	1.5482	0.0340	2.0
Rf	1.3060	0.0478	2.0
Rb1	3.8283	0.1218	2.0
Rc	0.7541	0.0203	2.0
Rb2	0.8886	0.0278	2.0
Rd	0.2478	0.0076	2.0

[a] Mass fraction values and uncertainties are listed with more significant digits than is scientifically warranted.

D. Potential Uncertainty Statement

The uncertainty provided with each value is an expanded uncertainty about the mean to cover the ginsenosides with approximately 95 % confidence. The expanded uncertainty is calculated as $U_k = k\,u_c$, where the combined uncertainty u_c incorporates the observed difference between the results from the methods and their respective uncertainties, consistently with the ISO Guide and with its Supplement 1, and k is a coverage factor corresponding to approximately 95 % confidence [D1-D3].

D. References

[D1] Joint Committee for Guides in Metrology, Evaluation of Measurement Data – Guide to the Expression of Uncertainty in Measurement, http://www.bipm.org/utils/common/documents/jcgm/JCGM_100_2008_E.pdf (2008) 1-120.
[D2] B. N. Taylor, C. E. Kuyatt, Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results, NIST Technical Note 1297, U.S. Government Printing Office: Washington, DC (1994); http://www.nist.gov/pml/pubs/tn1297/index.cfm.
[D3] B. Efron, R. J. Tibshirani, An Introduction to the Bootstrap, Chapman and Hall, UK, (1993).

Appendix E

SRM Preparation Documentation

E. NRC Canada Purity Measurements SRM 3389 Materials

National Research Council
Canada

Conseil national de recherches
Canada

Institute for National
Measurement Standards

Institut des étalons
nationaux de mesure

Ottawa, Canada
K1A 0R6

NRC·CNRC

February 27th., 2008

Certificate of Analysis for a Suite of Six Ginsenosides: Rg1, Re, Rb1, Rc, Rb2 and Rd
Prepared for Blaze Scientific Industries

Ginsenosides Re, Rb1, Rd and some of Rc were purified from *Panax quinquefolius* root
extract using a combination of: dispersive extraction, liquid-liquid partitioning, open and
closed column chromatography, crystallisation/precipitation, charcoal treatment and
filtration. Ginsenosides Rg1, Rb2 and some of Rc were obtained from Blaze Scientific
Industries and subject to further purification as described above without the extraction
and partitioning steps. Purity (Table 1) was determined by quantitative ^{1}H NMR using a
high purity benzoic acid (NIST SRM 350b) as an internal standard. No representation is
made as to the amount of substance of any impurities other than moisture or the stability
of the ginsenosides. The identity of all the ginsenosides was confirmed by ^{1}H and ^{13}C
NMR and mass spectroscopies (Tables 2 - 5).

Table 1. Quantity, Purity and Moisture Content for suite of Six Ginsenosides

Ginsenoside (labelled date)	net weight (g)	[1]Purity (%) ($\pm$ Uc$_{95}$, k=2)	[2]Moisture (%) ($\pm$ 1 s)
Rg1 (080128)	0.4081	97.2 $\pm$ 0.8	1.29 $\pm$ 0.18
Re (070907)	1.3764	98.0 $\pm$ 1.0	1.55 $\pm$ 0.19
Rb1 (080104)	3.0007	97.5 $\pm$ 0.6	2.27 $\pm$ 0.09
Rc (070907)	0.5368	96.4 $\pm$ 1.0	3.26 $\pm$ 0.23
Rb2 (080115)	0.4821	97.6 $\pm$ 0.9	1.83 $\pm$ 0.08
Rd (080108)	0.7096	95.3 $\pm$ 1.0	1.11 $\pm$ 0.03

[1] wet weight basis by ^{1}H qNMR, Uc$_{95}$ expanded uncertainty (2s), [2] Karl Fisher titration

Table 2. Accurate Mass Measurements

Ginsenoside	Formula	[1]Molar Mass (g.mol^{-1})	[2]Theoretical Molar Mass (g.mol^{-1})	[2]Measured Molar Mass (g.mol^{-1})	Error (ppm)
Rg1	$C_{42}H_{72}O_{14}$	801.01	823.4814	823.4811	-0.36
Re	$C_{48}H_{82}O_{18}$	947.15	969.5393	969.5391	-0.21
Rb1	$C_{54}H_{92}O_{23}$	1109.29	1131.5922	1131.5920	-0.18
Rc	$C_{53}H_{90}O_{22}$	1079.27	1101.5816	1101.5813	-0.27
Rb2	$C_{53}H_{90}O_{22}$	1079.27	1101.5816	1101.5812	-0.36
Rd	$C_{48}H_{82}O_{18}$	947.15	969.5393	969.5390	-0.31

[1] average molar mass, [2] mass charge ratio of monoisotopic (^{12}C,^{1}H,^{16}O) ion: [M+Na]$^+$

Canadä

Table 3a. ^{13}C NMR Spectroscopy, Suite of Six Ginsenosides

Carbon N	Rg1 (ppm)[1]	Re (ppm)[1]	Rb1 (ppm)[1]	Rc (ppm)[1]	Rb2 (ppm)[1]	Rd (ppm)[1]
1	40.6	40.4	40.7	40.7	40.7	40.7
2	27.7	27.4	26.1	26.0	26.0	26.0
3	78.1	78.3	91.4	91.4	91,5	91.4
4	40.5	40.5	40.4	40.4	40.4	40.3
5	61.9	61.5	57.6	57.6	57.7	57.6
6	78.3	79.9	18.2	18.1	18.1	18.1
7	45.4	46.2	35.8	36.0	36.1	36.0
8	42.0	42.1	41.1	41.1	41.1	39.7
9	52.5	52.6	52.2	52.5	52.5	52.6
10	40.3	40.4	38.0	38.0	38.0	38.0
11	31.1	31.0	31.4	31.4	31.6	31.1
12	71.3	71.3	70.3	71.7	71.1	71.3
13	50.4	50.5	50.6	50.7	50.8	50.7
14	53.2	53.2	53.0	53.0	53.0	53.3
15	31.5	31.9	30.9	30.9	30.9	31.8
16	26.0	26.6	26.1	26.0	26.0	26.0
17	52.5	52.6	53.0	53.0	53.0	53.3
18	17.7	17.8	16.8	16.4	16.8	17.0
19	17.2	17.6	16.4	16.8	16.8	18.1
20	85.1	85.1	85.1	85.9	85.1	85.1
21	22.9	22.9	22.6	22.5	22.5	23.0
22	36.7	36.7	36.9	36.9	36.9	36.8
23	24.3	24.4	24.0	24.0	24.0	24.4
24	126.0	125.9	126.17	126.1	126.1	126
25	132.4	132.4	132.29	132.3	132.4	132.4
26	26.0	26.0	27.4	27.4	27.4	27.4
27	17.9	17.3	17.5	19.4	17.5	19.4
28	31.1	32.1	28.5	28.5	28.5	27.5
29	17.4	17.4	16.8	16.8	16.4	16.8
30	17.2	17.4	17.3	18.1	17.5	16.4

[1] Relative to TMS in CD$_3$OD

Table 3b. ^{13}C NMR Spectroscopy, Suite of Six Ginsenosides (cont.)

Carbon N	Rg1 (ppm)[1]	Re (ppm)[1]	Rb1 (ppm)[1]	Rc (ppm)[1]	Rb2 (ppm)[1]	Rd (ppm)[1]
1'	105.7	101.7	105.5	105.5	105.5	105.5
2'	75.5	75.5	81.1	83.3	81.2	81.2
3'	79.2	79.2	77.2	77.8	78.0	78.0
4'	71.8	71.3	71.8	72.0	71.7	71.7
5'	78.3	78.1	77.8	78.5	78.5	78.0
6'	63.1	63.2	62.9	63.1	63.0	63.0
1"		101.7	104.6	104.6	104.7	104.6
2"		72.5	77.0	77.2	77.2	76.0
3"		72.3	78.0	78.2	78.8	78.5
4"		71.3	71.7	71.7	71.8	71.3
5"		79.2	77.8	78.1	78.5	78.6
6"		18.1	63.2	63.0	63.2	63.2
at C20						
1'''	98.2	98.4	98.2	98.1	98.2	98.4
2'''	75.5	75.0	75.2	75.4	75.4	75.5
3'''	79.2	79.9	79.2	79.0	79.0	79.0
4'''	71.5	78.3	70.3	71.7	71.8	71.3
5'''	78.1	78.2	77.0	76.4	76.4	78.0
6'''	62.7	62.6	70.3	68.3	69.1	62.6
1''''			105.14	110	104.6	
2''''			74.8	75.6	72.2	
3''''			78.2	78.7	73,8	
4''''			71.7	85.9	81.2	
5''''			78.2	63.2	66.0	
6''''			62.9			

[1] Relative to TMS in CD$_3$OD

Table 4a. ^{1}H NMR Ginsenosides Rg1, Re and Rb1

N carbon	Rg1 (ppm)[1]	Re (ppm)[1]	Rb1 (ppm)[1]
1	1.72; 1.04	1.72; 1.08	1.72, 1.0
2	1.9; 1.38	1.91; 1.35	1.9; 1.3
3	3.6 m	3.48 (d, 12.0)	3.18 m
4			
5	1.1 d (10.8)	1.12 d (10.8)	0.69 d (11.2)
6	4.15 dt (10.8; 3.0)	4.32 dt (11.0; 3.1)	1.52 m; 1.41
7	2.12 m; 1.62	1.8 dd (12.0; 32.8); 1.62 m	1.76 m; 1.5 m
8			
9	1.48	1.45	1.42
10			
11	1.84; 1.18	1.9; 1.18	1.75; 1.21
12	3.42	3.65 m	3.77
13	1.78	1.72	1.75
14			
15	1.58; 1.15	1.58; 1.02	1.55; 1.02
16	1.67; 1.58	1.62; 1.55	1.82; 1.32
17	2.28 m	2.3 m	2.30 m
18	1	1.0	0.88
19	1.08	1.1	0.93
20			
21	1.38	1.49	1.38
22	1.8; 1.61	1.8; 1.58	1.52; 1.25
23	2.05	2.31	2.1; 2.0
24	5.1 t (8.0)	5.12 t (8.5)	5.13 t (8.0)
25			
26	1.68	1.65	1.65
27	1.6	1.60	1.60
28	1.45	1.70	1.10
29	1.12	1.15	0.90
30	0.9	0.9	1.00

[1] Relative to TMS in CD_3OD

Table 4b. ^{1}H NMR Ginsenosides Rg1, Re and Rb1 (cont.)

Carbon N	Rg1 (ppm)[1]	Re (ppm)[1]	Rb1 (ppm)[1]
1'	4.3 d (8)	4.67 d (7.3)	4.4 d (7.5)
2'	3.2	3.6 m	3.55
3'	3.6	3.3	3.30
4'	3.68	3.7	3.60
5'	3.1	3.2	3.20
6'	3.8d; 3.6 m	3.85 dd; 3.65 m	3.8d; 3.6 m
1"		5.3	4.68 d (8.0)
2"		3.9 d	3.25 m
3"		3.68 m	3.55
4"		3.4 m	3.39
5"		3.1	3.15
6"		1.6	3.81d; 3.58 m
1'''	4.6 d (7.8)	4.6 d (7.3)	4.65 d (7.8)
2'''	3.1m	3.2	3.10
3'''	3.3 m	3.4	3.76
4'''	3.15	3.4	3.74
5'''	3.5 m	3.2 m	3.33
6'''	3.78 d; 3.6 m	3.75 dd; 3.6 m	4.1 dd(12.0; 3.0), 3.76
1''''			4.3 d (8.0)
2''''			3.10
3''''			3.55
4''''			3.70
5''''			3.23
6''''			3.81d; 3.58 m

[1] Relative to TMS in CD$_3$OD

Table 5b. ^{1}H NMR Ginsenosides Rc, Rb2 and Rd (cont.)

Carbon N	Rc (ppm)[1]	Rb2 (ppm)[1]	Rd (ppm)[1]
1'	4.98	4.45 d (7.5)	4.45 d (7.8)
2'	3.55	3.57	3.60
3'	3.30	3.34	3.30
4'	3.63	3.70	3.65
5'	3.20	3.12	3.20
6'	3.8d; 3.55 m	3.9, 3.5 m	3.8; 3.55 m
1"	4.65 d (8.0)	4.3 d (8.0)	4.65 d (8.0)
2"	3.20	3.20	3.20
3"	3.65	3.76	3.75
4"	3.45	3.73	3.70
5"	3.14	3.30	3.38
6"	3.79d; 3.62 m	3.70; 3.55 m	3.72; 3.6
1'''	4.55 d (8.0)	4.6 d (7.8)	4.6 d (8.0)
2'''	3.10	3.20	3.10
3'''	3.76	3.76	3.75
4'''	3.71	3.73	3.70
5'''	3.34	3.30	3.30
6'''		4.0 dd (12.0; 3.4), 3.7 m	3.72; 3.55
1''''	4.87	4.68 d (7.8)	
2''''	3.90	3.40	
3''''	3.50	3.50	
4''''	3.90	4.15	
5''''	3.78; 3.78	3.8 m; 3.5	
6''''			

[1] Relative to TMS in CD_3OD

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E: NRC Canada Purity Measurements for Calibrant Reference Standards

July 31st., 2011

Dear Christian,

Please find below a summary of the analytical data we have to date on the suite of ginsenosides shipped to you this week. I will be out of the lab now until Aug 22nd. Best regards, Tony

Summary, qNMR and TGA analyses of six ginsenosides batch AW110725

Component	Mass fraction (mg/g)	standard uncertainty (u) (mg/g)	expanded uncertainty (U) (mg/g)	TGA moisture (mg/g ±1s)
✓ Rg1	965	6	12	17.3 (0.4)
✓ Re	978	5	10	13.7 (1.0)
✓ Rb1	967	5	10	19.0 (0.3)
✓ Rc	925	6	12	61.9 (1.7)
✓ Rb2*	888	5	10	17.9 (3.1)
✓ Rd	897	7	14	75.3 (1.0)

(handwritten at left of table:) 22427-39-0 52286-51-6 41753-43-9 11021-14-0 11021-13-9 52705-93-8

qNMR was by external standards vs. NIST benzoic acid 350b, n=5 except Rb2 and Rc where n=3. qNMR mass fraction is on a wet weight basis. TGA moisture n=3 all cases determined from weight loss at 110°C

* Note a significant structural impurity was found in Rb2 sample and identified as a related ginsenoside Rb3 with a mass fraction by qNMR of 85 mg/g, u= 10 mg/g. Rb2 was the only sample with a measurable residue on ignition at 1.3 (0.6) mg/g.

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E. NRC Canada Certificate of Analysis for Ginsenoside CRM MIGS-1

Certificate of Analysis

**NRC·CNRC** — Certified Reference Material

MIGS-1

Mixed Ginsenoside Certified Reference Material

The following table show those constituents for which certified values have been established for this certified reference material (CRM). Certified values are based on results from data generated at NRC using quantitative proton nuclear magnetic resonance spectroscopy (^{1}H-qNMR).

The expanded uncertainty (U_{CRM}) in the certified value is equal to $U = ku_c$ where u_c is the combined standard uncertainty calculated according to the JCGM Guide [1] and k is the coverage factor. A coverage factor of two (2) was applied for all elements. It is intended that U_{CRM} accounts for every aspect that reasonably contributes to the uncertainty of the measurement.

Table 1: Certified quantity values in MIGS-1

Ginsenoside (a)	CAS Number	Molecular formula	Mass fraction, mg/g
Rb1	41753-43-9	$C_{54}H_{82}O_{23}$	1.249 ± 0.016
Rb2	11021-13-9	$C_{53}H_{90}O_{22}$	0.252 ± 0.002
Rb3	68406-26-8	$C_{53}H_{90}O_{22}$	0.023 ± 0.002
Rc	11021-14-0	$C_{53}H_{90}O_{22}$	0.505 ± 0.005
Rd	52705-93-8	$C_{48}H_{82}O_{18}$	0.507 ± 0.006
Re	52286-59-6	$C_{48}H_{82}O_{18}$	0.506 ± 0.002
Rg1	22427-39-0	$C_{42}H_{72}O_{14}$	0.258 ± 0.005
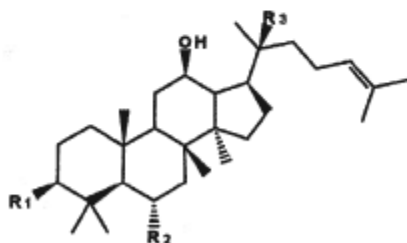
	R_1	R_2	R_3
Rg1	OH	1Glc	1Glc
Re	OH	1Glc(2-1)Rha	1Glc
Rb1	1Glc(2-1)Glc	H	1Glc(6-1)Glc
Rc	1Glc(2-1)Glc	H	1Glc(6-1)Araf
Rb2	1Glc(2-1)Glc	H	1Glc(6-1)Arap
Rb3	1Glc(2-1)Glc	H	1Glc(6-1)Xyl
Rd	1Glc(2-1)Glc	H	1Glc

Structure of ginsenosides
Standard IUPAC notation is used for monosaccharides

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All ginsenosides are identified here using their common acronyms and the corresponding Chemical Abstract Services (CAS) Registry Numbers. All measurements contributing to this certificate were conducted by Chemical Metrology personnel at NRC in Ottawa.

Coding

The coding refers to the instrumental method of analyte determination.

a   quantitative proton nuclear magnetic resonance spectroscopy (^{1}H-qNMR)

Certified values

Certified values are considered to be those for which NRC has the highest confidence in accuracy and that all known and suspected sources of bias have been taken into account and are reflected in the stated expanded uncertainties (Table 1).

Intended use

This reference material is primarily intended for use in the calibration of instrumentation, including LC-UV and LC-MS, for the quantitative and/or qualitative analysis of ginsenosides. The ginsenosides are certified on a mass fraction basis in methanol.

Conversion to mass concentration (mass per volume) may be done assuming that the density of the material is that of pure methanol. Note, however, that the density of methanol changes by 0.1% per degree Celsius (in the interval from 10 to 30 °C).

Storage and sampling

It is recommended that the material be stored in a cool, clean location at −20 °C temperature or lower.

Preparation of MIGS-1

Individual ginsenosides were isolated from four year old roots of North American ginseng *Panax quinquefolius L.* supplied by Chai-Na-Ta Corp. (Richmond BC, Canada) and grown at Harper Ranch (Kamloops BC, Canada). The identity of the roots was independently confirmed by Agriculture and Agri-Foods Canada and a voucher specimen has been deposited with the University of Ottawa herbarium (UO 19908). The roots were processed by Canadian Phytopharmaceuticals Corp. (Richmond BC, Canada). Following grinding to pass 80 mesh, the root material was extracted with aqueous ethanol, partially dried, mixed with a maltodextrin support and spray dried. Purification of the ginsenosides from the spray dried extract was done at NRC Ottawa using a combination of liquid-liquid partitioning, flash chromatography, multi-pass preparative HPLC and crystallisation.

Stability

The stability of MIGS-1 was tested using a two-week and four week isochronous stability test at +37 °C, +20 °C, +4 °C, and −20 °C temperatures with reference to samples held at −80 °C. All analytes were found to be stable.

Homogeneity

The ginsenosides are fully soluble in methanol at the concentrations prepared for MIGS-1 and therefore the material is deemed to be homogeneous.

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Uncertainty

The uncertainties of each integral were calculated according to the JCGM Guide [1] and combined using the DerSimonian-Laird (DSL) variance weighting procedure [2]. Included in the combined uncertainty estimate (u_c) are uncertainties in the batch characterization and uncertainties related to possible between-bottle variation and stability. However, given that homogeneity and stability components were deemed insignificant, the combined uncertainty is identical to the characterization uncertainty. The expanded uncertainty (U_{CRM}) in the certified values is equal to $U_{CRM} = 2u_c$ where u_c is the combined standard uncertainty.

Metrological traceability

Results presented in this certificate are traceable to the SI through gravimetrically prepared standards of high purity benzoic acid, SRM 350b, obtained from NIST which has established purity and supported by international measurement intercomparisons. As such, MIGS-1 serves as suitable reference material for laboratory quality assurance programs, as outlined in ISO/IEC 17025.

Quality management (ISO/IEC 17025, ISO Guide 34)

This material was produced in compliance with the documented NRC MSS Quality System, which conforms to the requirements of ISO/IEC 17025 and ISO Guide 34.

The Quality Management System supporting NRC calibration and measurement capabilities, as listed in the Bureau International des Poids et Mesures (BIPM) key comparison database (kcdb.bipm.org), has been reviewed and approved under the authority of the Inter-American Metrology System (SIM) and found to be in compliance with the expectations of the Comité International des Poids et Mesures (CIPM) Mutual Recognition Arrangement. The SIM certificate of approval is available upon request.

Updates

Users should ensure that the certificate they have is current. Our website at www.nrc.gc.ca/crm will contain any new information.

References

1. Evaluation of measurement data: Guide to the expression of uncertainty in measurement JCGM-100:2008.
2. R. DerSimonian, N. Laird (1986) Meta-analysis in clinical trials. Controlled Clinical Trials 7: 177-188

Authorship

The following staff members of the Measurement Science and Standards portfolio at NRC contributed to the production and certification of MIGS-1: Phuong Mai Le, Cathie Fraser, Juris Meija, Jennifer Bates, Anthony Windust, Patricia Grinberg and Zoltan Mester.

The cooperation of the following is gratefully acknowledged: Rana Zoka.

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MIGS-1

Date of issue: April 2015
Date of expiry: April 2020
Revised: March 2016 (editorial update)

Approved by:

Zoltan Mester, Ph. D.
Discipline Leader, Chemical Metrology
Measurement Science and Standards

This Certificate is only valid if the corresponding product was obtained directly from NRC or one of our qualified vendors.

Comments, information and inquiries should be addressed to:

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Également disponible en français sur demande.

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E. PhytoLab Certificate of Analysis for Ginsenoside Rf

the nature network®

PhytoLab

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Date: 7.01.16

Cust.No: 91790

Certificate of analysis

Report-No.:   20272627- 99 002
Batch: 10535
Article: 89213   Ginsenoside Rf
Quantity: 1 x 50 mg

Test	Unit	Limit	Testresult
Appearance, SOP 100005		powder	Conform
Color, SOP 100006		white	Conform
Solubility, SOP 105001:			Conform
Methanol		soluble	Conform
Identification (HPLC-HR/MS), SOP 204125		Conform	Conform
Identification (UV spectrum from HPLC-DAD analysis) according to specification, SOP 204311		Conform	Conform
Identification (IR-spectroscopy, Ph.Eur. 8.0, 2.2.24)/USP 37 <197>), SOP 206000		Conform	Conform
Identification (1H-NMR-spectroscopy), (outsourced), SOP 206010		Conform	Conform
Identification (13C-NMR-spectroscopy), (outsourced), SOP 206020		Conform	Conform
Water content, (micro determination, coulometric titration), Ph.Eur. 8.6, 2.5.32, SOP 304291:			
Mean value	%		2.0
Ginsenoside Rf, (HPLC), method 1, (% AU), SOP 400273	%	> = 90.00	98.01

Page   1 of   2

55

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PhytoLab

Certificate of analysis

Report-No.: 20272627- 99 002
Batch: 10535
Article: 89213 Ginsenoside Rf

Test	Unit	Limit	Testresult
Peakpurity, (HPLC), SOP 401367		Conform	Conform
Residual solvents, (headspace-GC), SOP 805765:			
Residual solvents	%		1.78
inorganic impurities, (ICP-MS), for reference substances, SOP 811701:			
Sodium	%		< 0.1
Potassium	%		< 0.1
Magnesium	%		< 0.1
Calcium	%		< 0.1
Aluminium	%		< 0.1
Phosphorus	%		< 0.1
Sulfur	%		< 1.0
Content*, SOP 890000	%		94

Assessment:
The above mentioned reference substance meets the specification.

*The absolute content is calculated considering the chromatographic purity, and if available, the content of water, residual solvents and inorganic impurities according to the following formula:
Content = (100% - water content (%) - residual solvents (%) - inorganic impurities (%)) x chromatographic purity (%) / 100.

The chromatographic purity is checked regularly: the last analysis has been performed in July 2015.

The reference substance cannot be documented with an expiry date. The pack is closed an is recommended to be stored as indicated. The unopened product is guaranteed to fulfill the specifications of this analytical report for a period of 60 months. Once opened we can no longer guarantee the stability of the material.

Vestenbergsgreuth, 7.01.16

Tamara Ackermann