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Validation of a Microscale Extraction and High Throughput UHPLC-QTOF-MS Analysis Method for Huperzine A in *Huperzia*: Microscale UHPLC-MS Analysis of Huperzine A

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Abstract

Herein we report on an improved method for the microscale extraction of huperzine A (HupA), an acetylcholinesterase-inhibiting alkaloid, from as little as 3 mg of tissue homogenate from the clubmoss *Huperzia squarrosa* (G. Forst.) Trevis with 99.95 % recovery. We also validated a novel UHPLC-QTOF-MS method for the high-throughput analysis of *H. squarrosa* extracts in only 6 min, which, in combination with the very low limit of detection (20 pg on column) and the wide linear range for quantification (20 to 10,000 pg on column), allow for a highly efficient screening of extracts containing varying amounts of HupA. Utilization of this methodology has the potential to conserve valuable plant resources.

Keywords

alkaloid; *Huperzia*; huperzine A; mass spectrometry; microscale extraction

Introduction

The traditional Chinese herbal medicine *Qian Ceng Ta*, a hot infusion prepared from whole plant material of the club moss *Huperzia serrata* (Thunb. ex Murray) Trevis, has been used for more than 1,000 years (earliest written record in 739 A.D.) for the treatment of swellings, contusions, neuromuscular disease and schizophrenia (reviewed in Ma *et al.*, 2007). *In vitro* and *in vivo* studies demonstrated that alkaloid-containing extracts from members of the *Huperziaceae* and *Lycopodiaceae* had inhibitory effects on acetylcholinesterase (AChE) (Cheng *et al.*, 1986; Tang *et al.*, 1989; Tang, 1996; Raves *et al.*, 1997). This enzyme is currently one of the most promising drug targets for the symptomatic treatment of cardiovascular and neuromuscular conditions, including Alzheimer's disease (AD). The active principles of *Huperzia serrata* extracts, the lycodine alkaloids huperzine A (HupA) and huperzine B, were isolated and identified in the 1980s (Liu and Zhu, 1986). HupA has undergone successful phase IV clinical trials in China, which indicated that it improves cognitive function in patients suffering from AD (Xu *et al.*, 1995; Xu *et al.*, 1999; Zhang *et al.*, 2002).

HupA occurs at low levels in members of the *Huperziaceae* (Ma *et al.*, 2005). Within this family the genus *Huperzia* consists of 10–15 species with temperate to arctic distribution,

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while the genus *Phlegmariurus* comprises primarily epiphytic species native to tropical and subtropical habitats. Several chemical syntheses of HupA have been published to date (reviewed in Ma *et al.*, 2007), but poor yields have excluded commercial applications and the primary source of HupA is still *H. serrata*. However, there are several challenges affecting this method of production. Species belonging to the *Huperzia* genus are known for their very slow growth rate of only a few centimeters per year and are difficult to propagate *in vitro* (Ma and Gang, 2008). Because of over-harvesting for medicinal preparations, *H. serrata* may soon become an endangered species (Ma *et al.*, 2006).

Species belonging to the *Huperziaceae* and *Lycopodiaceae* are known to accumulate large amounts of mannose-containing polysaccharides (Popper and Fry, 2004), which can interfere with the isolation of alkaloids. The combination of relatively low HupA quantities in *Huperzia* species and difficulties with its extractability have necessitated continuous improvements in analytical protocols (Ma *et al.*, 2005; Borloz *et al.*, 2006; Wu and Gu 2006; Pan *et al.*, 2006; Goodger *et al.*, 2008; Wang *et al.*, 2009; Zhang *et al.* 2009; Yu *et al.*, 2010). To further advance these efforts we have developed an optimized microscale extraction protocol for tissue extracts of *Huperzia squarrosa* (G. Forst.) Trevis, a club moss growing in temperate and tropical habitats of Southeast Asia and Australasia. Subsequent high-throughput analysis of HupA was achieved using an Ultrahigh Performance Liquid Chromatography system coupled to a Quadrupole Time-Of-Flight Mass Spectrometer (UHPLC-QTOF-MS).

Experimental

Reagents

Liquid chromatography solvents were obtained from EMD Biosciences (Gibbstown, NJ) and Burdick & Jackson (Morristown, NJ). Authentic HupA standard was obtained from Sigma-Aldrich (St. Louis, MO). The internal standard atropine was purchased from MP Biomedicals (Solon, OH).

Plant growth

Cuttings of *H. squarrosa* were obtained from the University of Colorado (greenhouse of the Department of Ecology and Evolutionary Biology). The cuttings were rooted in Perlite soil (Portland, OR) and grown under natural daylight, at an average humidity of 60 % and an average temperature of 23°C. A voucher specimen of *H. squarrosa* was deposited at the Field Museum at the University of Illinois (no. 2294678).

HupA extraction

Metabolite extractions were generally performed from tissue of actively growing sporophytes. Tissue was shock-frozen in liquid nitrogen immediately after harvest, ground to a powder using mortar and pestle, and dried to a constant weight in a freeze-drier. Roughly 3 mg of freeze-dried tissue was extracted with 2 mL of an aqueous acetic acid solution (2 %; v/v containing 2.7 µg/mL of the tropane alkaloid atropine, which does not occur in *H. squarrosa* and is used as a standard to calculate losses during the extraction procedure), by vigorous mixing for 10 min (Vortex mixer at highest speed) and subsequent sonication in an ultrasonic bath for 30 min at 23°C. The liquid phase was separated from tissue debris by spinning at 2,500 *x g* for 2 min. This extraction step was repeated with another 2 mL of 2 % acetic acid. The pooled supernatants were extracted once with 3 mL CHCl₃. The (lower) chloroform layer was discarded and the pH of the aqueous layer adjusted to pH 11 using 9 M aqueous ammonia. The basic aqueous solution was extracted twice with 3 mL CHCl₃, and the chloroform fractions were pooled and dried under reduced pressure. The residue was dissolved in 300 µL methanol and passed through a 0.45 µm

PTFE syringe filter (Millipore, Billerica, MA) directly into a 2.5-mL HPLC glass vial (Agilent Technologies, Santa Clara, CA). Extracts were placed on a chilled (4°C) autosampler and analyzed within 12 h. Variability of column loading was assessed by injecting one sample 10 times and was found to be negligible (< 2 % variation).

HupA analysis by UHPLC-QTOF-MS

HupA analyses were performed by high resolution mass spectrometry (MS) and tandem mass spectrometry (MS/MS) experiments using an Agilent 1290 UHPLC system coupled to an Agilent 6520 QTOF mass spectrometer equipped with a Atmospheric Pressure Chemical Ionization (APCI) source. Instrument control and data acquisition utilized MassHunter Acquisition Software (Revision B.02.01). MS and MS/MS data were processed and analyzed with Agilent's MassHunter Qualitative Analysis Software (Revision B.03.01). Chromatographic separations were achieved on a Zorbax Eclipse Plus C18 (2.1 × 50 mm, 1.8 μm particle size) analytical column. The temperature of the column compartment was kept at 60°C. The injection volume was typically set to 2 μL. The flow rate was 1.3 mL/min using 0.2 % aqueous acetic acid as solvent A and 0.2% acetic acid in methanol as solvent B. A linear gradient was applied from 98% A and 2% B to 2% A and 98% B at 3 min (plus an additional hold at 98% B for another 1 min). The column was re-equilibrated for 2 min between runs. The QTOF mass spectrometer was set to 2 GHz high gain in positive ion mode for MS experiments and 4 GHz high resolution mode for MS/MS experiments, with the following additional settings: the drying gas flow was 5 L min⁻¹, the nebulizer pressure was 60 psi, the drying gas temperature was 325°C, and the APCI source vaporizer temperature was 450°C. Scan rates were set to 1.4 scans s⁻¹ for MS and to 4 scans s⁻¹ for MS/MS. For fragmentation of HupA the [M+H]⁺ peak at m/z 243.149 was targeted. MS and MS/MS spectrometric peaks were annotated using accurate mass data for molecular formula generation in Agilent's MassHunter qualitative analysis software. Serial dilutions of the HupA standard were prepared from a 1 mg/mL stock solution in methanol, and the linearity of the QTOF mass spectrometer was assessed in the range of 0.01–100 μg/mL HupA, which corresponds to on-column injections of Hup A of 20–2,000 pg. A calibration curve with excellent linearity for the range of 0.01–50 μg/mL HupA (R² = 0.998) was used for quantification purposes.

Recovery

The recovery of HupA from 2% acetic acid extracts was evaluated using roughly 3 mg (dry weight) of pooled (scales and stems), finely ground, tissue samples of *H. squarrosa*. Three sets of five replicate tissue samples were prepared by (set 1) spiking with 2.37 μg of Huperzine A before extraction, (set 2) spiking 2.37 μg of Huperzine A after extraction, and (set 3) processing the tissue without spiking. Extracts (2 μL) were analyzed using a UHPLC-QTOF mass spectrometer. The recovery (**R**) was determined using the following equation:

$$R = 100 * \frac{\text{Peak Area (Sample Spiked Before Extraction)} - \text{Peak Area (Sample)}}{\text{Peak Area (Sample Spiked After Extraction)} - \text{Peak Area (Sample)}}$$

Matrix effects

To determine the effects of the biological matrix on the quantification of HupA, we extracted 3 mg (dry weight) of pooled (scales and stems), finely ground, tissue samples of *H. lucidula*, a close relative of *H. squarrosa* that does not accumulate detectable levels of HupA. These extracts were then spiked with HupA (final concentration of 0.01–50 μg/mL) and analyzed by UHPLC-QTOF-MS. These results were compared to those obtained with

neat standard solutions of the same concentration and the difference between these values was used to adjust peak areas for matrix effects.

Results and discussion

Development of a UHPLC-QTOF-MS method for huperzine A analysis

Three HPLC-MS-based methods for HupA analysis have been published previously (Wu and Gu, 2006; Borloz et al., 2006; Ma and Gang, 2008). Our initial optimization focused on shortening the chromatographic run time, while preserving the separation of HupA from potentially interfering metabolites contained in *H. squarrosa* extracts. A UHPLC gradient from 98% solvent A (0.2 % acetic acid) to 98 % solvent B (0.2 % acetic acid in methanol) using a porous, low particle-size, reversed-phase column operated at 1.3 mL/min enabled the analysis of HupA in *H. squarrosa* within only 6 min (incl. re-equilibration) (Fig. 1). Under these conditions the HupA authentic standard eluted at 0.6 min (retention factor $k' = 4.1$). An isocratic elution of HupA was unsuccessful, as *H. squarrosa* extracts contained a metabolite with a closely related m/z value that could only be separated by gradient HPLC. HupA did not ionize at all in APCI(−) mode and only a low signal was detected in ESI(−) mode. In ESI(+) mode the $[M+H]^+$ and the corresponding sodium adduct and dimers were detected with moderate sensitivity. In APCI(+) mode the $[M+H]^+$ ion was detected with exquisite sensitivity (17-fold higher abundance than in ESI(+) mode), while the sodium adduct and the protonated dimer were not detected (Fig. 1). All further analyses were thus performed in APCI(+) mode. MS/MS spectra of the HupA authentic standard and HupA present in *H. squarrosa* extracts had characteristic fragments at m/z 226.12 (loss of NH_3) and m/z 210.09 (Fig. 1).

The linear range of detection was from 0.01 to 50 $\mu\text{g/mL}$ ($R^2 = 0.998$; Table 1), corresponding to 20–100,000 pg injected on column. The limit of detection (signal-to-noise (S/N) ratio of 3:1) and the limit of quantification (S/N ratio of 10:1) were determined as 5.1 and 17.2 ng/mL, respectively. These values correspond to 10.2 and 17.2 pg injected on column, respectively (Table 1), which is a significant improvement over the most sensitive previously published protocols (Ma *et al.*, 2005; Zhang *et al.*, 2009). The intraday variation of the method, determined as the relative standard deviation (RSD) of replicate measurements of HupA was at 7.1% at a low concentration (0.1 $\mu\text{g/mL}$) and decreased to 1.3% at higher concentrations (50 $\mu\text{g/mL}$). The RSD for interday precision ranged between 6.5 and 7.6% for lower concentrations (0.1–10 $\mu\text{g/mL}$) and was lower (3.7%) at the highest concentration (50 $\mu\text{g/mL}$).

Impact of matrix effects on huperzine A quantification by UHPLC-QTOF-MS

When separations using HPLC or UHPLC are combined with MS or MS/MS detectors, matrix effects can lead to ion suppression or enhancement, thus leading to potentially incorrect quantifications of target analytes. To evaluate the relevance of matrix effects for the detection of HupA in *H. squarrosa*, we used extracts from a closely related species, *H. lucidula*, which did not contain any detectable amounts of HupA. These extracts were spiked with known concentrations of HupA and the obtained peak areas in UHPLC-QTOF-MS analyses compared to those acquired analogously with neat HupA standards of equivalent concentrations. The peak area difference was then used to determine matrix effects according to established methods (Matuszewski *et al.*, 2003). The slopes of both curves (spiked extracts and neat standard) were nearly identical (Fig. 2). The only significant difference was that the HupA peak intensity dropped near the limit of detection in the presence of the *Huperzia* matrix. In the presence of a *Huperzia* matrix, the limit of detection was determined as 10.1 ng/mL or 20.2 pg (42.1 fmol on-column) and the limit of quantification was 33.7 ng/mL or 67.4 pg (141.9 fmol) on-column.

Recovery of huperzine A from *H. squarrosa* extracts

HupA was extracted using a standard protocol with an initial acid treatment of finely ground tissue samples, an extraction with CHCl_3 to remove potentially interfering metabolite, an alkalization of the extract to protonate HupA, and a second extraction with CHCl_3 to transfer the now protonated HupA into the organic phase (Ma *et al.*, 2005). The only differences to this traditional protocol were a few adjustments for a smaller scale extraction and the use of 2 % acetic acid instead of 2 % tartaric acid, as this resulted in extracts containing fewer metabolites potentially interfering with a rapid and sensitive HupA analysis (data not shown). For the calculation of extraction recoveries we used a standard method validation protocol that is based on a spiking approach (Hartmann *et al.*, 1998). Samples were analyzed in three ways using UHPLC-QTOF-MS: (i) by acetic acid extraction without modifications ('Sample' column in Table 2); (ii) by adding a known quantity of authentic HupA standard to the extraction solvent prior to tissue processing ('Sample Spiked Before Extraction' column in Table 2); and (iii) by adding a known quantity of authentic HupA standard to the extract after completing the tissue extraction ('Sample Spiked After Extraction' column in Table 2). These experiments were performed with 3 mg (dry weight) of *H. squarrosa* tissue, which is approximately 1/7 the tissue amount required for the least tissue-consuming protocol published to date (Ma and Gang, 2008). The recovery for HupA was 99.95%, indicating only marginal losses during the extraction (Table 2).

Quantification of huperzine A in *H. squarrosa*

Using our newly developed and validated UHPLC-QTOF-MS protocol the HupA concentration in greenhouse-grown *H. squarrosa* sporophytes was determined to be 415.3 $\mu\text{g/g}$ dry weight (Table 3), which is very similar to previously published values for field specimen harvested in China (378.8 $\mu\text{g/g}$; Ma *et al.*, 2005). We also separated scales and stems prior to the extraction and obtained HupA concentrations of 928.0 and 317.7 $\mu\text{g/g}$ dry weight, respectively. These results indicate that scales are a roughly 3-fold richer source of HupA compared to stems, which had not been evaluated previously. These results have implications for studies on the biosynthesis of HupA, as the tissue-level localization of the biosynthetic pathway is currently unknown.

Conclusions

Our new UHPLC-QTOF-MS method for the analysis of HupA from *Huperzia* offers advanced with regard to sensitivity (and a concomitant decrease in the amount of required plant material) and chromatographic run time. This new method is highly valuable for rapidly determining HupA concentrations in *Huperzia*, even when plant material is limited.

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References

- Borloz A, Marston A, Hostettmann K. The determination of Huperzine A in European *Lycopodiaceae* species by HPLC-UV-MS. *Phytochemical Analysis*. 2006; 17:332–336. [PubMed: 17019934]
- Cheng YS, Lu CZ, Ying ZL, Ni WY, Zhang CL, Sang GW. 128 Cases of *Myasthenia gravis* treated with huperzine A. *Chinese Journal of New Drugs and Clinical Remedies*. 1986; 5:197–199.
- Goodger JQD, Whincup AL, Field AR, Holtum JAM, Woodrow IE. Variation in huperzine A and B in Australasian *Huperzia* species. *Biochemical Systematics and Ecology*. 2008; 36:612–618.

- Hartmann C, Smweyers-Verbeke J, Massart DL, McDowall RD. Validation of bioanalytical chromatographic methods. *Journal of Pharmaceutical and Biomedical Analysis*. 1998; 17:193–218. [PubMed: 9638572]
- Liu J-S, Zhu Y-L. The structures of huperzine A and B, two new alkaloids exhibiting marked anticholinesterase activity. *Canadian Journal of Chemistry*. 1986; 64:837–839.
- Ma X, Gang DR. *In vitro* production of huperzine A, a promising drug candidate for Alzheimer's disease. *Phytochemistry*. 2008; 69:2022–2028. [PubMed: 18538805]
- Ma X, Tan C, Zhu D, Gang DR. A survey of potential huperzine A natural resources in China: the Huperziaceae. *Journal of Ethnopharmacology*. 2006; 104:54–67. [PubMed: 16203116]
- Ma X, Tan C, Zhu D, Gang DR. Is there a better source of Huperzine A than *Huperzia serrata*? Huperzine A content of Huperziaceae Species in China. *Journal of Agricultural and Food Chemistry*. 2005; 53:1393–1398. [PubMed: 15740012]
- Ma X, Tan C, Zhu D, Gang DR, Xiao P. Huperzine A from *Huperzia* species – An ethnopharmacological review. *Journal of Ethnopharmacology*. 2007; 113:15–34. [PubMed: 17644292]
- Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*. 2003; 75:3019–3030. [PubMed: 12964746]
- Pan J, Jin R, Hu X. Application of L-amino acid in determination of huperzine A by high performance liquid chromatography. *Journal of Chromatography B*. 2006; 836:108–110.
- Popper ZA, Fry SC. Primary cell wall composition of pteridophytes and spermatophytes. *New Phytologist*. 2004; 164:165–174.
- Raves ML, Harel M, Pang YP, Silman I, Kozikowski AP, Sussman JL. Structure of acetylcholinesterase complexed with the nootropic alkaloid, (–)-huperzine A. *Nature Structural Biology*. 1997; 4:57–63.
- Tang XC, De Sarno P, Sugaya K, Giacobini E. Effect of huperzine A, a new cholinesterase inhibitor, on the central cholinergic system of the rat. *Journal of Neuroscience Research*. 1989; 24:276–285. [PubMed: 2585551]
- Tang XC. Huperzine A shuangyiping: a promising drug for Alzheimer's disease. *Zhongguo Yao Li Xue Bao*. 1996; 17:481–484. [PubMed: 9863136]
- Wang G, Cao Q, Zhu X, Yang X, Yang M, Ding Z. Molecular imprinted solid-phase extraction of Huperzine A from *Huperzia serrata*. *Journal of Applied Polymer Science*. 2009; 113:3049–3058.
- Wu Q, Gu Y. Quantification of huperzine A in *Huperzia serrata* by HPLC-UV and identification of the major constituents in its alkaloid extracts by HPLC-DAD-MS-MS. *Journal of Pharmaceutical and Biomedical Analysis*. 2006; 40:993–998. [PubMed: 16337768]
- Xu SS, Cai ZY, Qu ZW, Yang RM, Cai YL, Wang GQ, Su XQ, Zhong XS, Cheng RY, Xu WA, Li JX, Feng B. Huperzine A in capsules and tablets for treating patients with Alzheimer's disease. *Zhongguo Yao Li Xue Bao*. 1999; 20:486–490. [PubMed: 10678137]
- Xu SS, Gao ZX, Weng Z, Du ZM, Xu WA, Yang JS, Zhang ML, Tong ZH, Fang YS, Chai XS, et al. Efficacy of tablet huperzine A on memory, cognition, behavior in Alzheimer's disease. *Zhongguo Yao Li Xue Bao*. 1995; 16:391–395. [PubMed: 8701750]
- Yu L, Shi Y, Huang J, Gong Y, Liu Z, Hu W. Modification and validation of a high-performance liquid chromatography method for quantification of huperzine A in *Huperzia crispa*. *Journal of AOAC International*. 2010; 93:1428–1435. [PubMed: 21140653]
- Zhang Y, Xie J, Chen W-Q, Zhou T-Y, Lu W. Development of a sensitive high-performance liquid chromatographic method with simple extraction for simultaneous determination of huperzine A and huperzine B in the species containing Lycopodium alkaloids. *Journal of AOAC International*. 2009; 92:1060–1063. [PubMed: 19714972]
- Zhang Z, Wang X, Chen Q, Shu L, Wang J, Shan G. Clinical efficacy and safety of huperzine alpha in treatment of mild to moderate Alzheimer disease. A placebo-controlled, double-blind, randomized trial. *Zhonghua Yi Xue Za Zhi*. 2002; 82:941–944. [PubMed: 12181083]

Fig. 1. Identification of HupA in *H. squarrosa* extracts. (A) Total Ion Current (TIC) and (B) Extracted Ion Chromatograms (EIC) obtained by UHPLC-QTOF-MS in APCI (+) mode for the HupA standard (red trace) and the 1 % acetic acid extract (black trace). HupA elutes at 0.62 min. (C) MS and (D) MS/MS spectra of the *H. squarrosa* extract at 0.62 min. (E) MS and (F) MS/MS spectra of the authentic HupA standard at 0.62 min. The following fragments were identified in the MS/MS spectra of the extract: (1) $243.1477 = [M+H]^+$, (2) 226.1225 (loss of NH_3), and (3) 210.0890 (unannotated).

Fig. 2.

Evaluation of matrix effects on HupA detection by UHPLC-QTOF-MS. The blue line indicates the calibration curve with neat HupA standard ($N = 6$), while the red line indicates measured peak areas for known quantities of HupA spiked into an extract obtained from *H. lucidula*, a close relative of *H. squarrosa* that does not contain detectable levels of HupA ($N = 6$). Standard deviations are indicated, but are mostly smaller than the symbols used to plot the mean of the measurements.

Table 1

Validation of a UHPLC-QTOF-MS method for the analysis of HupA.

Retention Factor	Linearity	R ²	LOD (S/N = 3:1)	LOQ (S/N = 10:1)	Intraday Precision* (N = 6) (RSD [%])	Interday Precision* (N = 5) (RSD [%])
Values for neat standard solutions						
4.1	0.01 – 50 µg/mL	0.998	5.1 ng/mL	17.2 ng/mL	0.1 µg/mL	1 µg/mL
	20 – 100,000 pg (OC)		10.2 pg (OC)	34.4 pg (OC)	50 µg/mL	10 µg/mL
					1.3	7.0
					3.4	7.6
					1.3	3.7
Values for neat standard solutions in the presence of <i>aHyperzia</i> matrix						
4.1	0.015 – 50 µg/mL	0.997	10.1 ng/mL	33.7 ng/mL	0.1 µg/mL	1 µg/mL
	30 – 100,000 pg (OC)		20.2 pg (OC)	67.4 pg (OC)	50 µg/mL	10 µg/mL
					1.3	7.0
					3.4	7.6
					1.3	3.7

LOD, Limit Of Detection; LOQ, Limit Of Quantification; OC, Amount Injected On Column; S/N, Signal-to-Noise Ratio.

Table 2

Recovery of HupA from *H. squarrosa* extracts. Presented values reflect normalized peak areas.

Replicate	Sample (3 mg)	Sample Spiked Before Extraction	Sample Spiked After Extraction
1	534319	1108856	1105289
2	658796	1081894	1014307
3	668484	1031501	1025945
4	647848	1081574	1137156
5	621004	1073785	1096033
Mean	626090	1075522	1075746
SD	54287	27970	53175
RSD [%]	8.7	2.6	4.9
R [%]	99.95		

R, Recovery; RSD, Relative Standard Deviation; SD, Standard Deviation.

Table 3Distribution of HupA in different tissues of *H. squarrosa*.

Hup A Concentration (Dry Weight) [$\mu\text{g/g}$]			
	Sporophyte (N = 8)	Scales (N = 6)	Stems (N = 6)
Mean	415.3	928.0	317.7
SD	29.6	40.8	28.0
RSD [%]	7.1	4.4	8.8

RSD, Relative Standard Deviation.

SD, Standard Deviation.