HHS Public Access

Author manuscript

Food Chem. Author manuscript; available in PMC 2019 October 30.

Published in final edited form as:

Food Chem. 2018 October 30; 264: 449–454. doi:10.1016/j.foodchem.2018.04.093.

Quantitative HPLC-MS/MS Analysis of Toxins in Soapberry Seeds: Methylenecyclopropylglycine and Hypoglycin A

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Abstract

Methylenecyclcopropylglycine (MCPG) and hypoglycin A (HGA) are naturally occurring amino acids found in various soapberry (*Sapindaceae*) fruits. These toxins have been linked to illnesses worldwide and were recently implicated in Asian outbreaks of acute hypoglycemic encephalopathy. In a previous joint agricultural and public health investigation, we developed an analytical method capable of evaluating MCPG and HGA concentrations in soapberry fruit arils as well as a clinical method for the urinary metabolites of the toxins. Since the initial soapberry method only analyzed the aril portion of the fruit, we present here the extension of the method to include the fruit seed matrix. This work is the first method to quantitate both MCPG and HGA concentrations in the seeds of soapberry fruit, including those collected during a public health investigation. Further, this is the first quantitation of HGA in litchi seeds as well as both toxins in mamoncillo and longan seeds.

Keywords

Soapberry Seeds; *Sapindaceae*; Hypoglycemic Encephalopathy; Hypoglycin A (HGA); Methylenecyclopropylglycine (MCPG)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript

Disclaimer

The findings and conclusions in this study are those of the authors and do not necessarily represent the views of the U.S. Department of Health and Human Services, the U.S. Centers for Disease Control and Prevention, or the U.S. Department of Agriculture. The use of trade names and commercial sources is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services, the U.S. Centers for Disease Control and Prevention, or the U.S. Department of Agriculture. The authors declare no competing financial interest.

1. Introduction

Methylenecyclcopropylglycine (MCPG) and hypoglycin A (HGA) are amino acids that have been shown to cause hypoglycemia and encephalopathy (Melde, 1991; Tanaka, 1976). During a recent public health investigation, these toxins were linked to human cases of acute hypoglycemic encephalopathy (Shrivastava et al., 2017). The Centers for Disease Control and Prevention (CDC) provided laboratory support for this investigation by developing two detection methods to determine potential exposure to MCPG and HGA (Shrivastava et al., 2017). The toxins can be found in common soapberry (*Sapindaceae)* fruits including ackee (*Blighia sapida*), litchi (*Litchi chinensis*), longan (*Dimocarpus longan*), and mamoncillo (*Melicoccus bijugatus*) (Das et al., 2015; Isenberg et al., 2015; Isenberg et al., 2016; John et al., 2014; Paireau et al., 2012; Pulla, 2015; Shrivastava et al., 2017; Shrivastava et al., 2015; Spencer et al., 2014; Yadav, 2015). The first method developed, a clinical method, evaluated the presence of the toxin metabolites in urine (Isenberg et al., 2015). The second method, an agricultural method, was jointly developed with the United States Department of Agriculture (USDA) and analyzed the levels of both toxins in soapberry fruit arils (Isenberg et al., 2016).

Earlier studies of ackee (*B. sapida*) fruit, a member of the soapberry family, showed elevated levels of HGA in the seed as compared to the edible aril of the fruit (Bowen-Forbes et al., 2011; Brown et al., 1991). Ackee (*B. sapida*) arils are generally safe to consume when ripe, but it is not recommended to consume the ackee seeds. Toxin levels in the seeds of soapberry fruits other than ackee (*B. sapida*) have neither been quantified nor compared to the toxin levels in their respective arils. In the present work, we expand our previously published agricultural method for aril matrices to also quantify MCPG and HGA in soapberry seed matrices. Following development, the method was used to quantify MCPG and HGA in the seeds of rambutan (*Nephelium lappaceum*), litchi (*L. chinensis*), longan (*D. longan*), and mamoncillo (*M. bijugatus*) fruit using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS).

2. Materials and Methods

2.1. Safety

Appropriate PPE should be worn at all times while working with MCPG, HGA, and chemical standards. Both MCPG and HGA are known to cause hypoglycemia upon ingestion.

2.2. Materials

IsoSciences, LLC (King of Prussia, PA, USA) was contracted for the synthesis of isotopically-labeled (≥99.5 %, $^{13}C_3$ -MCPG* and $^{15}N^{13}C_2$ -HGA*) and unlabeled (≥ 97 %, MCPG and HGA) standards. Dansyl chloride (98 %) and all solvents (acetonitrile, methanol, water-HPLC grade) were purchased from Fisher Scientific (St. Louis, MO, USA). Ethanol (≥ 99.5 %), formic acid (98 %), sodium hydroxide (0.1 N), and 10X phosphate buffered saline were purchased from Sigma-Aldrich (Pittsburgh, PA, USA). Laboratory deionized water (18 MΩ-cm) was generated in house using an Aqua Solutions Water Purification

system (Jasper, GA, USA). Oasis HLB 96-well 5 mg per well solid phase extraction plates were acquired from Waters Technologies Corporation (Milford, MA, USA).

2.3. Fruit Extraction

The method used for extracting fruit arils (n=50) from five genera of the soapberry family was previously published and modified in this work (Fig. S1). (Isenberg et al., 2016) Soapberry seeds were separated from the outer coat (Fig. S2). Seed samples were obtained with biopsy forceps (Surgical Tools, Inc. P/N 66.23.10). Samples were dehydrated at 57 °C for one hour using a Nesco FD-75PR Snackmaster Pro Food Dehydrator (Two Rivers, WI, USA), and then 1.0–3.0 mg portions were weighed in homogenizer tubes using Mettler Toledo AG204 (Columbus, OH, USA). The Omni Bead Ruptor 24 (Kennesaw, GA, USA) was used to homogenize the biopsied samples in 80 % ethanol solution at 4200 rpm for 30 seconds (x 2) with a 2 minute dwell time at room temperature. A 400 μL aliquot of 80 % ethanol in deionized water (v/v) was added to each homogenizer tube. The homogenate was centrifuged for 2 minutes at 15,800 × g using an Eppendorf Centrifuge 5415R (Hauppauge, NY). The supernatant was then transferred into a 96 deep-well plate. The same biopsy sample was extracted three more times to ensure extraction efficiency. Each extraction supernatant was transferred to a separate well in the 96 deep-well plate. The supernatants were then dried under nitrogen at 60 °C for one hour. For an initial 2 mg seed, the four supernatant wells each received 250 μL of deionized water and was then combined to be 1 mL. The workflow for this preparation is illustrated in Fig. S1 with the resulting 2 mg/mL seed in water prepared for analysis.

2.4. Sample Preparation

Sample preparation following extraction of the fruit was previously published and now includes both the aril and the seed. (Isenberg et al., 2016) Briefly, isotope dilution was carried out in a 96 deep-well plate followed by dansyl chloride derivatization and then solid phase extraction (SPE). After SPE, each well was dried under nitrogen at 60 °C for 25 minutes. The well residues were resuspended in 50 μL of 0.1 % formic acid in deionized water prior to analysis by HPLC-MS/MS. Calibrators were prepared at concentrations of 1.00, 2.00, 5.00, 10.0, 20.0, 50.0, 100., and 200. ng/mL MCPG and HGA in water. The internal standard solution was prepared at a concentration of 100. ng/mL MCPG* and HGA* in deionized water. When prepared, these materials correspond to 1.00 – 200. μg/g according to the conversion below.

$$\frac{\mathrm{ng}_{\mathrm{analyte}}}{\mathrm{mL}_{\mathrm{solution}}} \times \frac{\mathrm{mL}_{\mathrm{solution}}}{\mathrm{mg}_{\mathrm{seed}}} = \frac{\mathrm{ng}_{\mathrm{analyte}}}{\mathrm{mg}_{\mathrm{seed}}} = \frac{\mu\mathrm{g}_{\mathrm{analyte}}}{\mathrm{g}_{\mathrm{seed}}}$$

During sample preparation, 25 μL matrix blank was added to the deep well plate with calibrators and internal standard. The matrix blank was 2 mg/mL rambutan (*N. lappaceum*) seed extract (n=5, pooled) in water that showed no endogenous levels of toxin. Quality control (QC) materials were prepared in matrix blank at concentrations of 3.50, 15.0, and 75.0 μg MCPG and HGA per gram of dried seed. All materials were stored at ≤ −20 °C prior to use.

2.5. HPLC-MS/MS

The HPLC-MS/MS parameters were previously published but modified in this work. (Isenberg et al., 2016) MCPG and HGA toxin levels in soapberry seeds were determined on an AB Sciex 4000 triple quadrupole mass spectrometer (Framingham, MA, USA) using positive-mode ESI. Conventional HPLC elution was performed using an Agilent 1260 Infinity series chromatograph (Santa Clara, CA, USA). Samples were injected at 4.0 μL volumes onto an Agilent Zorbax SB-C18 Rapid Resolution HT column (2.1 × 50 mm, 1.8 μm) equipped with an Agilent low dispersion in-line filter (2 μm frit) (Santa Clara, CA, USA). The column and autosampler tray temperatures were 60 °C and 5 °C, respectively. Mobile phase A consisted of 0.1% formic acid in HPLC grade water. Mobile phase B consisted of 0.1 % formic acid in HPLC grade acetonitrile. The gradient was delivered at 500 μL/min starting with 10 % mobile phase B for 0.10 minutes, then ramped to 70 % over a period of 2.40 minutes. A high organic wash was added for 0.25 minutes at 90 % mobile phase B, followed by equilibration of the column at 10 % mobile phase B for 1.24 minutes. The instrument parameters were as follows: collision gas, 7 psig; curtain gas, 10 psig; ion source gas 1 and 2, 60 psig; ion spray voltage, 4500 V; temperature, 500 °C; collision exit potential, 5.0 V; dwell time, 75.0 ms, unit resolution, 0.7 amu at full half width max; dns-MCPG/dns-MCPG* declustering potential, 45 V; entrance potential, 8.0 V; dns-HGA/dns-HGA* declustering potential, 40 V; entrance potential, 12 V). Quantitation was carried out using multiple-reaction monitoring (MRM) (dns-MCPG quantitation ion *m/z* 361.1 → 170.1, confirmation ion *m/z* 361.1 → 157.1; dns-MCPG* *m/z* 364.1 → 157.1; dns-HGA quantitation ion *m/z* 375.1 → 170.1, confirmation ion *m/z* 375.1 → 157.1; dns-HGA* *m/z* 378.1 → 170.1) The collision energies were as follows: dns-MCPG quantitation ion 29 V, confirmation ion 39 V; dns-MCPG* 39 V; dns-HGA quantitation ion 27 V, confirmation ion 39 V; dns-HGA* 27 V.

2.6. High Resolution Mass Spectrometry

A Thermo Scientific Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (Waltham, MA, USA) was used to acquire high mass accuracy product ion spectra. Fragmentation was accomplished using MS/MS HCD at 35 normalized collision energy 30,000 resolution, and 1.5 *m/z* isolation width.

2.7. Data Acquisition and Processing

Analyst v.1.6 (AB Sciex, Framingham, MA, USA) was used for data acquisition and quantitative analysis. Percent relative error ($\%\ \mathrm{RE} = \left[\frac{(C_e - C_t)}{C_t}\right] \times 100\ \%$ where C_e is experimental concentration and C_t is the theoretical concentration) and percent relative standard deviation ($\%\ \mathrm{RSD} = \left[\frac{\mathrm{SD}}{C_{avg}}\right] \times 100\ \%$ where SD is the standard deviation, and C_{avg} is the average concentration calculated) were calculated to determine method precision and accuracy. This method was characterized using CDC Multi-Rule Quality Control System (MRQCS) with no more than two curves per day by four analysts over four weeks (Caudill et al., 2008).

2.8. Results Reporting and Application Sample Set

For each dried seed, the concentration of analyte is provided in ng analyte/mL solution, but can be converted to μg analyte/g dried seed (Isenberg et al., 2016). The seeds of five Rambutans (*N. lappaceum*), five longans (*D. longan*), six mamoncillos (*M. bijugatus*), one ackee (*B. sapida*), and twenty-six litchis (*L. chinensis*) were analyzed using the method described herein. This convenience set tested included the seeds from the same soapberry fruit whose arils were tested in the previously reported method, and the fruits were purchased between May 2015 and May 2016 (Isenberg et al., 2016). An additional group of litchis (*L. chinensis*) was included in this study that was purchased in June 2016 commercially in the United States. Canned fruit were not tested in this method because they are sold without seeds. Samples for which a laboratory analysis was requested during a hypoglycemic encephalopathy outbreak included thirty-two litchi seeds that were collected from May to June 2014 (Shrivastava et al., 2017).

3. Results and Discussion

3.1. Detection and Separation

The method analytes, dansylated (dns-) MCPG and HGA, elute chromatographically at 2.73 ± 0.12 minutes and 2.89 ± 0.11 minutes (n=20), respectively. The confirmation ion ratios for MCPG and HGA are 0.91 ± 0.35 and 0.77 ± 0.07, respectively. Initial matrix effects were evaluated to determine whether rambutan (*N. lappaceum*) seed would be a suitable matrix blank for the method calibrators. To do this, a 200. ng/mL solution of dns-MCPG and dns-HGA in water was infused while injecting 1 mg/mL matrix blank, and no matrix effects were observed at the expected retention times (Fig. S3) (Annesley, 2003; Isenberg et al., 2016). The peak signal intensity of the lowest calibrator was three-fold higher than the matrix blank for both analytes (Fig. 1), with a lowest reportable limit (LRL) of 1.00 ng/mL (1.00 μg/g dried seed). For MCPG in seed, the theoretical LOD was calculated with the Taylor method to be 0.831 ng/mL, and the theoretical LOD for HGA in seed was 0.676 ng/mL (Taylor, 1987). The highest reportable limit (HRL) corresponds to 200. μg/g in seed. In addition to the method calibration range (1–200 ng/mL), dilution experiments were evaluated at concentrations up to 20 μg/mL (20 mg/g dried seed). The matrix blank was enriched with known levels of MCPG and HGA and then diluted to fit into the calibration curve with ≤ 17 % error (Table 1).

3.2. Precision, Accuracy, and Linearity

Precision, accuracy, and linearity were evaluated during the characterization of the seed matrix method for MCPG and HGA (Table 2). The MCPG and HGA R^2 values were 0.998 ± 0.001 and 0.998 ± 0.001, respectively. The % RE and % RSD were < 15 % for all calibrators. QC materials in matrix blank were monitored over the characterization and fell within the FDA foods and veterinary guidelines for the validation of chemical methods for accuracy and precision measurements of < 15 % (FDA, 2015).

3.3. Stability of QC Materials

Stability of the QC materials in matrix blank was evaluated at 4 °C, 22 °C, and 60 °C. The materials at 4 °C and 22 °C were evaluated for 24 hours, which corresponds to storing materials in a refrigerator overnight (4 °C) and to leaving materials out on a laboratory benchtop (22 °C). The stability of QC materials at 60 °C was evaluated for four hours in order to ensure analyte stability during sample preparation. Each sample undergoes heated dehydration and derivatization steps up to 60 °C for a maximum of 70 minutes. All stability results for both MCPG and HGA under storage conditions at 4 °C, 22 °C, and 60 °C were within 18% error relative to the characterized QC concentrations in Table 2, indicating that the analytes are stable under the conditions tested. The stability of QC materials stored in multi-use freezer vials was evaluated for 20 successive freeze-thaw cycles to ensure that materials remained useable throughout their stored volume (20 aliquots of 500 μL use). Experimentally-determined QC concentrations for both MCPG and HGA after 20 freeze-thaw cycles were less than 14% error relative to the concentration of the same QC after 0 freeze-thaw cycles.

3.4. Sampling, Extraction, and Analytical Ruggedness

The ruggedness of soapberry seed sample preparation was evaluated by analyzing intra-seed sample precision (1.0–3.0 mg) at pre- and post-homogenization biopsy stages. A small sample mass allows for a low volume of extraction solvent (1.6 mL total), and a larger amount was not needed due to the sensitivity of the quantitative HPLC-MS/MS analysis. In order to determine whether the small sample size was representative of the entire seed, an intra-seed biopsy ruggedness was evaluated using six replicate biopsies from a single seed and the relative standard deviation was ≤ 16 % for both MCPG and HGA (Table 3).

Initial development was a single extraction of the seed, but comparing intra-seed data resulted in a high % RSD. MCPG and HGA within a single seed had % RSDs of 43 % and 48 %, respectively. In order to ensure extraction efficiency, each seed sample was extracted four times. Increasing the number of extractions to four lowered % RSDs to 16 % and 15 %, respectively. The ruggedness of the extraction procedure was evaluated next by altering the following parameters: percent ethanol, homogenization time, and centrifugation time (Fig. S4). To do this, a litchi (*L. chinensis*) seed with previously quantified analyte concentrations was selected. Each ruggedness parameter was evaluated at lower and higher values (n=3) and compared to the optimized method conditions. The percent ethanol was varied from 60 % to 100 %. The homogenization time was varied from 30 seconds to 90 seconds, and the centrifugation time from one minute to three minutes. As plotted in Fig. S4, the average concentrations of MCPG and HGA for each varying parameter are within one standard deviation of each other. Therefore, the varying extraction parameters yielded no significant difference as compared to the characterized method parameters.

The analytical ruggedness was evaluated for QCs (high, medium, low) by varying the LC column temperature, injection volume, LC flow rate, SPE sorbent lots, and multiple column lots. The column temperature was varied to 50 °C and 70 °C. The injection volume was varied to 3.0 and 5.0 μL. The LC flow rate was varied to 400 and 600 μL/min. Multiple SPE sorbent lots and column lots (n=3) were incorporated in the characterization runs. All

experimentally-determined QC concentrations for both MCPG and HGA for the analytical ruggedness testing were within 16% error relative to the characterized QC concentration in Table 2. This confirmed the expected results due to isotope dilution.

3.5. Application of Method

The developed method was used to quantify toxin levels in a convenience set of common soapberry fruit seeds. The convenience set consisted of 5 rambutans (*N. lappaceum*), 5 longans (*D. longan*), 6 mamoncillos (*M. bijugatus*), 1 ackee (*B. sapida*), and 30 commercially purchased litchis (*L. chinensis*) (litchi groups 1–5, Table 4). A set of 32 litchi seeds obtained during the previously reported public health investigation (litchi group 6, Table 4 & Fig. 2) were also analyzed. Of the 30 litchi seeds from groups 1–5, only 26 were tested because four litchis had “chicken tongue seeds”, which are small aborted seeds that often contain a higher portion of aril with no seed inside of the coat (Fig. S2) (Z.Y. Yang, 2014). MCPG was observed in all litchi seeds tested and in some of the other seeds. HGA was observed in all soapberry seeds tested, except for rambutan (*N. lappaceum*). Ranges for each soapberry fruit seed are provided (Table 4).

A wide range of MCPG and HGA concentrations were observed in the various fruit seeds, ranging from < LRL (1 μg/g) to over 3000 μg/g. Although there are no regulatory restrictions on MCPG or other soapberry fruits, the importation of ackee fruit to the United States is restricted to fruit containing less than 100 μg/g of hypoglycin A (FDA, 2014). Consumers familiar with soapberry fruits are not expected to ingest the seed; however, a young child or unfamiliar consumer may be exposed to portions of the seed, which may lead to unsafe levels of toxins. For example, based on the maximum tolerated dose (MTD) of HGA observed in rats of 1.5 mg/kg, a 20 kg child would have an estimated MTD of 0.33 mg/kg, and would only need to ingest approximately 8 mg of HGA to exhibit symptoms of toxicity (Blake et al., 2006; FDA, 2002). As such, only half of a five gram seed (~3000 μg/g HGA) would be needed to reach the MTD. For MCPG, a MTD has not yet been reported in the literature, however, it is known to be “powerfully hypoglycemic” (Melde, 1991). Additionally, the additive toxic effects of MCPG and HGA together have not been investigated, so it is unknown whether the total MTD may be different when the two toxins are co-occurring as was observed in several of the seeds analyzed in this study. In a public health investigation in which an illness is believed to be related to soapberry toxin ingestion, the concentrations of MCPG and HGA in the arils and seeds of the fruit should be considered in conjunction with the symptomatic profile.

An isomer of HGA was observed in longan (*D. longan*) and mamoncillo (*M. bijugatus*) seeds at a retention time of 2.61 minutes and was baseline resolved from HGA. The presence of this isomer and HGA was previously reported in longan (*D. longan*) seeds (Minakata, 1985) and longan (*D. longan*) arils (Isenberg et al., 2016), and this work is the first observation of HGA and its isomer in mamoncillo (*M. bijugatus*) seeds. The high-resolution identification of this isomer and theoretical exact mass product ion spectrum are shown (Fig. S5). This isomer is believed to be 2-amino-4-methylhex-5-ynoic acid based on the earlier findings of Minakata and coworkers (Minakata, 1985).

Litchi (*L. chinensis*) seeds from six different sources were compared (Fig. 2), and all levels of MCPG and HGA in tested litchi seeds were above the LRL (1.00 μg/g dried seed). In the previously reported litchi method, toxin concentrations in the aril were significantly different between the commercially obtained convenience set and fruit obtained during the public health investigation (Isenberg et al., 2016). In the current work, similar concentration ranges were observed in all of the litchi seeds, including those obtained during the previous public health investigation. Due to a small sample size in the public health investigation, a statistical evaluation could not be made even though no difference was observed between the unripe and ripe seeds. The effects of ripening on MCPG in soapberry fruit are limited (Das et al., 2015). The ripeness of litchi with respect to HGA concentrations have not been reported; however, several reports of HGA in the seed and aril during ackee (*B. sapida*) maturation have suggested that seed levels remain constant while the concentration in the aril drops (Bowen-Forbes et al., 2011; Brown et al., 1991). Using this new method, investigators may now determine how MCPG and HGA levels vary with ripeness in both arils and seeds of soapberry fruit (Bowen-Forbes et al., 2011; Brown et al., 1991).

Conclusion

The method presented in this work expands the laboratory support capabilities for investigations into the presence of MCPG and HGA toxins, while conveniently using an analytical instrument platform previously developed. This work is the first to report MCPG and HGA concentrations in mamoncillo (*M. bijugatus*) and longan (*D. longan*) seeds. Additionally, this is the first work to report the presence of both toxins in litchi (*L. chinensis*) seeds. Concentrations of MCPG and HGA greater than the LRL (1.00 μg/g) were quantified in 100 % of the litchi seeds tested, with HGA being quantified in litchi seeds for the first time. Of note, litchi seeds obtained commercially did not differ significantly from those obtained during the public health investigation. In the future, this method may allow researchers to determine how ripeness affects the level of toxins between the soapberry family seed and aril matrices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

This work was supported by the Centers for Disease Control and Prevention, the Oak Ridge Institute for Science and Education, the Battelle Memorial Institute, and the U.S. Department of Agriculture.

The authors acknowledge Scott Landvatter, Rich Tyburski, and Stephen Jones for their efforts towards the initial custom synthesis that led to the commercial availability of the native standards and isotopically-labeled standards that were imperative for this work

Abbreviations

ave	average concentration

CDC	Centers for Disease Control and Prevention
dns-Cl	dansyl chloride
dns-HGA	dansyl hypoglycin A
dns-HGA*	dansyl-$^{15}N_{13}C_2$-hypoglycin A
dns-MCPG	dansyl-methylenecyclopropylglycine
dns-MCPG*	dansyl-$^{13}C_3$-methylenecyclopropylglycine
DI	deionized
ESI	electrospray ionization
FDA	U.S. Food and Drug Administration
HGA	hypoglycin A
HGA*	$^{15}N_{13}C_2$-hypoglycin A
HPLC–MS/MS	high-pressure liquid chromatography–tandem mass spectrometry
HRL	highest reportable limit
ISTD	isotopically labeled calibrator solution
LOD	limit of detection
LRL	lowest reportable limit
MCPG	methylenecyclopropylglycine
MCPG*	$^{13}C_3$-methylenecyclopropylglycine
MRM	multiple-reaction monitoring
MRQCS	multirule quality-control system
PPE	personal protective equipment
QC	quality control
% RE	percent relative error
% RSD	percent relative standard deviation
SD	standard deviation
SPE	solid-phase extraction

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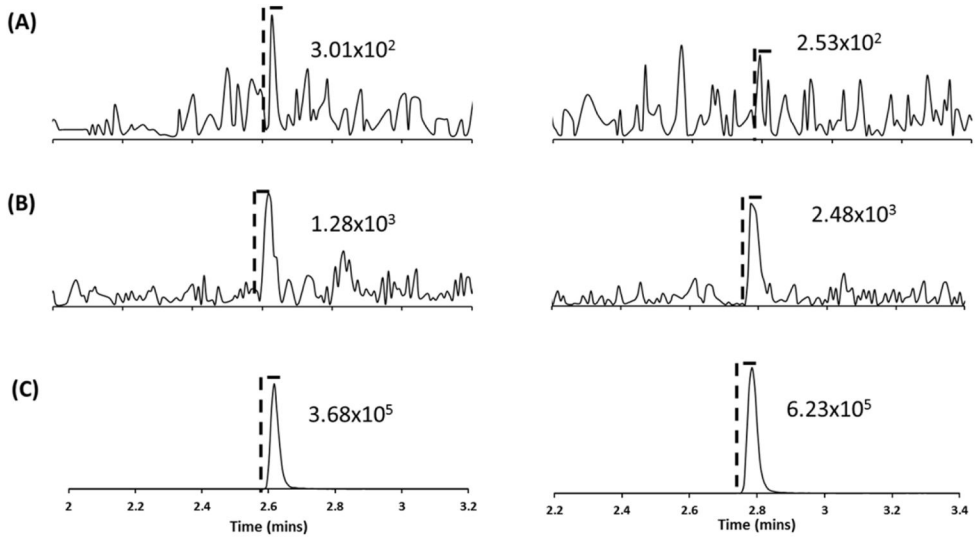

Figure 1.
Extracted Ion Chromatograms of Rambutan Seed Extract

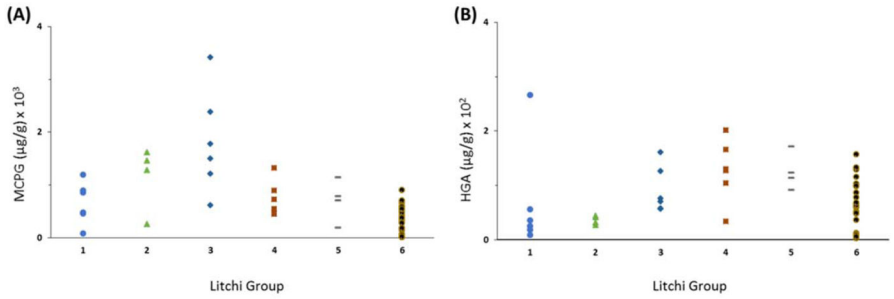

Figure 2.
Application of the method to commercial and investigation litchi fruit.

Table 1(A)

Dilution accuracy and precision of MCPG in rambutan seed extract up to 20.0 mg/g dried seed (n=3).

Theoretical (mg/g)	Experimental (mg/g)	stdev	% RE	% RSD
1.00	1.14	0.08	14	7.0
5.00	5.38	0.21	7.7	3.9
10.0	9.27	1.28	−7.3	14
20.0	17.9	0.7	−10	3.8

Table 1(B)

Dilution accuracy and precision of HGA in rambutan seed extract up to 20.0 mg/g dried seed (n=3).

Theoretical (mg/g)	Experimental (mg/g)	stdev	% RE	% RSD
1.00	1.17	0.05	17	4.1
5.00	5.70	0.18	14	3.2
10.0	9.48	1.43	−5.2	15
20.0	16.7	0.6	−17	3.7

Table 2

Precision (% RSD) and Accuracy (% RE) of QC and calibrator characterization (n=20).

	MCPG		HGA	
Concentration (ng/mL)	**% RSD**	**% RE**	**% RSD**	**% RE**
1.00	10	−11	15	6.1
2.00	9.6	15	5.3	−3.4
5.00	6.5	−0.70	7.2	1.7
10.0	7.4	−1.7	5.7	3.0
20.0	5.3	−8.9	6.8	−4.5
50.0	6.0	5.9	6.2	8.7
100.	3.1	2.5	3.1	3.8
200.	2.5	−1.9	2.7	−3.9
75.0 (QH, μg/g)	9.2	3.6	7.4	5.0
15.0 (QM, μg/g)	7.2	5.4	7.5	13
3.50 (QL, μg/g)	15	5.8	13	6.8

[*] Quality control (QC) materials were prepared in 2 mg/mL rambutan seed extract (matrix blank) at concentrations of 3.50, 15.0, 75.0 μg MCPG and HGA per gram of dried seed

Table 3

Soapberry seed sampling ruggedness between biopsies.

Fruit Sample*	MCPG (μg/g)**	HGA (μg/g)
1	1410	130
2	1490	186
3	1150	146
4	1780	178
5	1340	141
6	1720	179
Average ± std dev	**1480 ± 240**	**160. ± 20**
% RSD	**16**	**15**

*All biopsy samples were taken from a single litchi. Samples were dehydrated. From the dehydrated fruit, samples ranging from 1.0 to 3.0 mg were homogenized in 80 % ethanol.

**All MCPG results were from samples diluted 50X

Table 4

Application of method to quantify MCPG and HGA in soapberry seeds.

	MCPG (μg/g dried seed)	HGA (μg/g dried seed)
Rambutan (n=5)	< LRL-1.88	< LRL
Longan (n=5)	< LRL-5.91	45.3–3540
Mamoncillo (n=6)	<LRL-5.20	90.5–970
Ackee (n=1)	< LRL	7.72
Litchi Group 1 (n=6)	85.7–1190	8.68–267
Litchi Group 2 (n=4)	263–1620	27.4–41.2
Litchi Group 3 (n=6)	615–3420	57.2–161
Litchi Group 4 (n=6)	446–1320	34.1–202
Litchi Group 5 (n=4)	192–1140	91.5–172
Litchi Group 6 (n=32)*	8.72–905	2.57–157

*Group six consisted of litchi seeds obtained during a laboratory technical assist for the public health investigation. The LRL for both toxins is 1.00 μg/g dried seed.