Tracking toxic hypoglycin A over two maturity stages of different positions in four Chinese litchi cultivars by UPLC-MS/MS

Weili Tao

Shantou University Medical College

Yu Yang

Jinan University

Qiongjin Wang

Shantou University Medical College

Dong Chen

guangdong weian testing technology co., ltd

Chuanyi Zhao

Shantou University Medical College

Qian Lv

Shantou University Medical College

Yicun Chen (✉ chenyicun@yeah.net)

Shantou University Medical College

Research

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Abstract

Background

Litchi (*Litchi chinensis* Sonn.), a member of Sapindaceae family, is a common fruit with deliciously fragrant, sweet flavors and high commercial value, besides edible arils, the pericarps and seeds of fruits and the leaves of litchi all exhibited meaningful bioactivities, while as the bibliography reported, hypoglycin A (HGA) as a toxic amino acid was naturally occurring in some members of Sapindaceae family.

Methods

In this work, an UPLC-MS/MS method was developed to quantified HGA in different parts of four litchi cultivars which at two maturity stages. Meanwhile, the methodological indicators of the established method were evaluated by selectivity, linearity, precision, accuracy, recovery and stability.

Results

The consequences expressed that the levels of HGA were highly associated with litchi cultivars and maturity stages. The positions of seeds and branches were the major source of HGA in the four litchi cultivars been detected, as for the edible arils, especially of 'Fenghua' and 'Linglan' were relative safety to be taken in.

Conclusion

This developed method can provide scientific technical support for relevant research of content determination and this founding can offer scientific data for the further research of human health that was related to litchi cultivars.

Introduction

Litchi (*Litchi chinensis* Sonn.) as the member of the Sapindaceae family, is a tropical and subtropical tree originated from South-East Asia, and now widely cultivated in Asia, East Africa, Oceania, some Pacific Islands and so on [1, 2]. The fruit of litchi is globose or oblong to ovate with squamous speckle, red pericarp, white translucent fleshy aril, with appealing taste and sweet aroma, nevertheless, it is perishable [3]. As the literature reported, the aril of litchi possessed plenty of biological activities, containing antioxidant, prevent and anti-cancer, antimicrobial, antiviral, anti-inflammatory, anti-diabetic and immunomodulatory activities, all of these bioactivities was attributed to it is rich in various phytochemicals, including flavonoids, polysaccharides, polyphenol, fatty acids, lignans, anthocyanins, proanthocyanidins, coumarins, sterols and terpenes [4]. In addition, the litchi leaves extracts expressed anticancer cell proliferation, antioxidant [5], alleviate hepatic injury [6], antinociceptive [7] and anti-inflammatory properties [8]. As for the extracts from litchi pericarps and seeds, they exhibited the properties of anti-inflammatory, antioxidant, anti-glycated, tyrosinase inhibitory, anti-cancer and immunomodulatory [9].

Besides of these bioactive components, litchi also contains amino acids, such as hypoglycin A (HGA) and methylenecyclopropylglycine (MCPG), which as fruit-based toxins will cause hypoglycaemia and metabolic derangement, and finally evolve into the high mortality disease of acute neurological disorders [10, 11]. The mechanism of HGA and MCPG induce hypoglycemia is that they can conjugate to CoA by branches-chain oxo-acid dehydrogenase via α -oxidation and oxidative decarboxylation and then form methylenecyclopropylacetyl-CoA and methylenecyclopropylformyl-CoA, respectively. Then as a toxic metabolite can react with the flavin of general acyl-CoA dehydrogenase and lead to a direct and irreversible inhibition of β -oxidation of fatty acids, which will disturb the metabolism of glucose and result in hypoglycemia in the end [12, 13]. It was reported that during May to July, there were many children infected unexplained neurologic illness in India [10]. Under the study of [11] and [14], HGA and MCPG were regarded as the origin of this acute hypoglycemic encephalopathy. Actually, not merely in India, in other litchi-cultivating areas, such as China, Vietnam, Bangladesh and other Asia countries, this acute encephalopathy was occurred [14]. Therefore, in this study, not only did we determine the concentrations of HGA in different part of litchi, including leaves and branches of litchi tree and pericarps, edible aril and seeds of litchi fruit, but we also researched the relationship of HGA contents between different species of litchi.

Ultra high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) as a high resolution and sensitivity technique has been widely used in the nature product research, including the separation, identification and quantification of complex plant extraction, the applications in pharmacokinetics and biosystematics, the research of secondary metabolite biogenesis and structure elucidation, and so on [15]. HPLC-MS/MS has been applied in the quantification of HGA and MCPG in soapberry arils and seeds, in human plasma and in ackee fruits [16–19].

The aim of our study was to track the quantification of HGA in the pericarps, edible aril and seeds of litchi fruits which at different maturity stages and to compare the content of HGA in the branches, leaves and fruit of litchi, meanwhile, we also drew a comparison between various species of litchi. What should be emphasized is that all of these were accomplished by the high sensitivity analytical instrument UPLC-MS/MS.

Methods

Materials

The leaves, branches and fruits of four cultivars 'Linglan', 'Fengchuiliao', 'Fenghua' and 'gualv' of Litchi were collected from orchards in Jieyang, Guangdong Province of China. The fresh leaves, branches and fruits were selected with normal color and without obvious injuries.

All reagents used in the UPLC-MS/MS analysis were of HPLC grade. Methanol and formic acid were purchased from Macklin (Shanghai, China), ammonium acetate was bought from Kermel (Tianjing, China), HGA was obtained from TRC (Toronto, ON, Canada).

Standard preparation

Standard HGA was dissolved in methanol and prepared at a series concentrations of 10, 20, 50, 100, 200, 500 ng/mL.

Sample preparation

As shown in Fig.1, fresh fruits were divided into the parts of pericarps, edible arils and seeds and then all of these parts and leaves, branches were ground into powder by an YF-114B grinder (Yongli Pharmaceutical Machinery Co., Ltd, Zhejiang, China), these sample powder were stored at -18 °C. 1 g leaves, branches, pericarps and seeds powders were distributed in 15 mL centrifuge tubes and mixed with 5 mL methanol, respectively. Similarly, 1 g arils powders were suspended in 5 mL of methanol/water (10:90, v/v) mixture. Multireax oscillator (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) was used to extract HGA from samples at 1200 rpm for 3 h. After that, the extract solution was centrifuged at 10,000 rpm for 5 min by the using of H1850 centrifuge (Cence Laboratory Instrument Exploitation Co., Ltd, Hunan, China). In the end, the supernatant was collected and storage at -18 °C before the analysis of HPLC-MS/MS.

UPLC-MS/MS analysis

The qualitative and quantitative analyses of HGA were determined on a UPLC-MS/MS 8045 system (Shimadzu, Kyoto, Japan), which equipped with a triple quadrupole mass spectrometer using positive ionization mode and coupled with a UPLC equipped with a Shimadzu Shim-pack GIST-HP C18 column (2.1× 100 mm, 3 µm). Mobile phase A consisted of 0.1% formic acid and 10 mmol/L ammonium acetate in HPLC-grade water, mobile phase B composed of HPLC-grade methanol. The flow rate was 0.3 mL/min and the elution gradient was programmed as follows: 0 min, 22% B; 1 min, 22% B; 3 min, 25% B; 6 min, 30% B; 8 min, 60% B; 13 min, 80% B; and finally, the initial conditions were held for 3 min to re-equilibrate the column. In addition, the instrument parameters were set as follows: sheath gas flow was 3 L/min, aux gas flow was 10 L/min and heated gas flow was 10 L/min. The temperature of ion transfer tube, DL and heating block were 300, 250 and 400 °C, respectively. Multiple-reaction monitoring (MRM) was used to obtain the quantitative and qualitative data. The quantitation ion of HGA was based on the transition m/z 142.10 → 74.05, with regard to the conformation ion, was on the grounds of the transition m/z 142.10 → 96.05. The collision energies (CE) of HGA were 11.0 V.

Method validation

Linearity range, selectivity, sensitivity, precision, accuracy, stability and recovery were used to assess the validation of the method we used [20].

A series of standard solutions were dissolved in methanol at 10, 20, 50, 100, 200, 500 ng/mL for HGA. The standard curve was built with the mass concentration as the abscissa and the peak areas of quantification ion as the ordinate. Meanwhile, the correlation coefficient (R^2) was utilized to response the fitting degree of curvilinear regression equation [21]. As for selectivity, it was investigated by comparing the retention time of HGA in the samples. In addition, the sensitivity of this method was characterized by two parameters, limits of detections (LOD) and limits of quantifications (LOQ). LOD was assessed as the concentration of analyte that with signal-to-noise (S/N) ratio of 3:1, simultaneously, LOQ with S/N ratio of 10:1 [22].

The values of precision and accuracy were determined through the calculation of percent relative error (%RE) and percent relative standard deviation (%RSD) which with 6 separate measurements for three calibrators [16]. Furthermore, the stability of HGA was evaluated by three QC samples which were stood for 2 h, 4 h, 8 h, 12 h, 24 h at 4 °C on the same day (intra-day) and stored for 1, 3, 7, 15 and 30 days (inter-day) at -20 °C [23].

Extraction recovery was confirmed by comparing the average HGA concentrations of samples which were chosen from four litchi species data randomly with the average HGA concentrations of samples that were prepared by removing half volume of chosen litchi samples and then spiked with corresponding volumes of validation solutions representing 100% recovery [24]. The samples chosen from four litchi species were the branches of 'Fengchuiliao', the maturity pericarps of 'Fenghua', the immaturity seeds of 'Gualv' and the immaturity seeds of 'Linglan', respectively, and six replicates were carried at different samples.

Statistical analysis

The statistical analysis was calculated by SPSS version 20.0 in this work. ANOVA analysis and Independent-Samples T Test were used. Data was expressed as means ± standard deviation of triplicate detections. The determined data were owned significant differences. The p -value < 0.05 represented that the results were significant, and p -value < 0.01 on behalf of the results were very significant.

Results

Detection and separation

Compared with the analytic system gas chromatography-mass spectrometry (GC-MS) which samples needed to be derivatized [25, 26], in this work, samples were prepared by briefly steps of ultrasonic extraction and centrifugation without any chemical derivatization. As for the detection, it was performed by UPLC-MS/MS (Fig. 2), at a CE of 11.0 V, the precursor ion of HGA at m/z 142.10 in the positive mode was switched into quantitation ion at m/z 74.05 and conformation ion at m/z 96.05.

Selectivity, Linearity and sensitivity

Selectivity was a parameter been set to confirm the method specific to the detection of the unknown compounds. It was able to be assessed through comparing the retention time between standards and samples [27]. As demonstrated in Fig. 3, the retention time of HGA in the standards and determinands were both around 1.3 min In addition, throughout all the analysis process, the retention time without being observed a significant offset, which hinted that this method was fitted to the analysis of HGA with great selectivity.

The linearity of HGA was evaluated by standard substance being dissolved into methanol in the range of 10-500 µg/L. The standard curve was established as with $R^2 = 0.9992$. The correlation coefficient ($R^2 > 0.99$) represented that in the concentration range between 10 µg/L and 500 µg/L, the linearity was ideal [28].

Sensitivity was estimated by LOD and LOQ, the amount of LOD was three times as much as the peak height of baseline background, while for LOQ, it was ten times. LOD and LOQ can also be calculated through the slope of linear equation and the standard deviation of noise of the baseline background, the equation as follow [29].

See equation 1 in the supplementary files.

where σ represents the standard deviation of the response, and a on behalf of the slope of the standard curve.

According to the concentration observed at an S/N greater than three, the LOD value of the method was 6 µg/L or 0.03 µg/g when converted unit, meanwhile, the LOQ value of the method was 18 µg/L (0.10 µg/g). As Fig.3 shown, the peak signal intensity of the matrix blank was almost 15-fold less than that of the lowest standard point, which implied that the value of LOD of this method was rather low and this method was appropriate for the analysis of HGA in the litchi.

Precision, accuracy and recovery

As Table 1 demonstrated, three validation samples with the concentrations of 10 µg/L, 150 µg/L and 3500 µg/L respectively, were detected six times to determine the precision (%RSD) and accuracy (%RE) values of this method for HGA. The results shown that the %REs of validation samples were -0.17%, 0.02% and 0.14%, respectively, with corresponding %RSDs were 1.37%, 1.32% and 1.44%. Meanwhile, the mean of these concentrations were not exceeding 15% of the nominal values and the precision were within 15% [30].

According to the results of HGA contents in the litchi parts which were chosen to evaluate the extraction recovery, the concentrations of analyte solutions added to the sample solutions were as followed: 500 µg/L, 40 µg/L, 2000 µg/L and 4000 µg/L standard solutions were mixed with the sample solutions of 'Fengchuiliao', 'Fenghua', 'Gualv' and 'Linglan' chosen above in the equivalent volume, respectively. On the basis of the request, the allowable bias range of the extraction recovery is from 85% to 115%, and the RSD of extraction recovery should within 15% [31]. As Table 2 exhibited, 104.16%, 106.64%, 98.45% and 101.60% corresponding to the recovery of 'Fengchuiliao', 'Fenghua', 'Gualv' and 'Linglan' and all of these data were in the range between 85% to 115%, simultaneously, the corresponding %RSD were 2.18%, 3.49%, 3.03% and 2.98% which were all less than 15%.

Stability

HGA was proven to be stable in QC samples under different storage conditions at low, medium and high concentration levels. As shown in Table 3, the relative error was less than 3.07% for low, medium and high QC levels whether stored intra-day or inter-day, namely the bias of HGA concentrations in QC samples were within 15% of the theoretical concentrations [32].

Detection of HGA in four litchi cultivars

The HPLC-MS/MS method was applied to quantify the HGA contents of four litchi species 'Linglan', 'Fengchuiliao', 'Fenghua' and 'Gualv' at two degree of maturity in different parts including pericarps, arils, seeds, leaves and branches. The consequences were exhibited in Table 4. At the cultivar level, 'Fengchuiliao' showed the highest HGA concentrations in immature pericarps, immature arils, immature seeds and leaves. In addition, the mature arils and mature seeds also expressed rather high contents of HGA. As for the HGA level of 'Fenghua', the mature seeds and branches parts hinted maximum, it is notable that the branches of 'Fenghua' displayed the maximal HGA concentration among these data which was up to 114.165 ± 1.456 µg/g, while it was reported that the toxic dose of HGA for male and female rats was 231.19 ± 62.55 mg/kg BW [33]. Converting to a 50 kg adult, the toxic dose was as high as 101.252 kg which suggested that all the positions of these four litchi cultivars were relatively safe. The remaining positions of litchi, mature pericarps and mature arils, were revealed highest HGA concentrations in 'Gualv'. With regard to 'Linglan', the HGA contents in any part were lower than that of other species. Apart from the immature seeds of 'Linglan' was 15.092 ± 0.217 µg/g, the rest parts were less than 1.000 µg/g, that implied that in comparison with other cultivars, 'Linglan' expressed the less toxicity. Moreover, ANOVA analysis was employed to found the significant differences of various litchi cultivars. As Table 4 displayed, either in variety of parts or in different maturity stages, the concentrations of HGA were exhibited high correlation with litchi cultivars. From the perspective of position level, Independent-Samples T Test was applied to ensure whether the HGA concentrations of two maturity stages in various positions were significant or not, the consequences shown in Table 4 with p -value < 0.01, represented that the HGA concentrations in different maturity stages exist quite great differences. The consequences exhibited that the maximum HGA contents of mature and immature pericarps, mature and immature arils, mature and immature seeds, leaves and branches were 1.653 ± 0.036 µg/g, 0.869 ± 0.012 µg/g, 16.076 ± 0.161 µg/g, 25.231 ± 0.247 µg/g, 54.325 ± 0.747 µg/g, 35.140 ± 0.421 µg/g, 1.532 ± 0.024 µg/g and 114.165 ± 1.456 µg/g, severally.

Discussion

Litchi (*Litchi chinensis* Sonn.) as a member of the Sapindaceae family is widely cultivated in Asia, East Africa, Oceania, some Pacific Islands and so forth [34]. As the literature reported [4, 5, 9], not only the litchi owns edible arils, but also possesses the bioactive compounds in its pericarp and seed of fruits, leaves and branches. However, just like the other species in this family, it caused numerous public health issues in various counties, such as India, Vietnam, Bangladesh, and so on [14]. Actually to date [10], HGA has been deemed to relate to the deaths caused by Sapindaceae family. Results of this study confirm that HGA exists in the pericarp, seed and aril of fruits, leaves and branches.

The consequence showed that the branches of 'Fenghua' displayed the maximal HGA concentration which was up to $114.165 \pm 1.456 \mu\text{g/g}$. Actually, contrasted with other plants containing HGA, such as ackee with at least $271 \pm 58 \mu\text{g/g}$ HGA in arils and $893 \pm 65 \mu\text{g/g}$ HGA in seeds [19], maple tree had $856 \pm 677 \mu\text{g/g}$, $1365 \pm 795 \mu\text{g/g}$ and $31 \pm 49 \mu\text{g/g}$ HGA in seeds, sprouts and leaves separately [35], sycamore owned approximately $64.47 \mu\text{g/g}$ HGA in seeds, $43.3 \mu\text{g/g}$ HGA in leaves and $1210 \mu\text{g/g}$ HGA in seedlings [36], the litchi cultivars that detected in this work were relatively safer intake.

HGA as a specific inhibitor of isovaleryl CoA dehydrogenase and α -methylbutyrate dehydrogenase has been identified as the cause of atypical myopathy in horse and Père David's deer [37, 38], and Jamaican vomiting sickness, viral encephalitis and neurologic illness in human [39, 40]. In the cases of these diseases, extreme hypoglycemia was observed [10]. Our work implied that seeds and branches were the major source of HGA in the four litchi cultivars been detected, while the edible arils, especially of 'Fenghua' and 'Linglan' were relative safety to be taken in.

Conclusions

In this work, a simple and straightforward sample preparation method, using the UPLC-MS/MS, was established to detect the concentrations of HGA in different positions (Pericarps, arils, seeds, leaves and branches) of four litchi cultivars, Linglan', 'Fengchuiliao', 'Fenghua' and 'gualv', and to monitor HGA concentrations in different maturity stages of litchi fruits. According to the consequences of methodological indicators, the established method expressed great selectivity, linearity, precious, accuracy, recovery and stability, represented that this method is suitable for the analysis of HGA in litchi. As a result, the levels of HGA were highly associated with litchi cultivars and maturity stages. In addition, the branches of 'Fenghua' displayed the maximal HGA concentration which was up to $114.165 \pm 1.456 \mu\text{g/g}$, by the way, the positions of seeds and branches were the major source of HGA in the four litchi cultivars been detected, as for the edible arils, especially of 'Fenghua' and 'Linglan' were relative safety to be taken in. Actually, this developed method can provide scientific technical support for relevant research of content determination and can offer scientific data for the further research of human health that was related to litchi cultivars.

Abbreviations

CE: collision energies; HGA: hypoglycin A; LOD: limits of detections; LOQ: limits of quantifications; MRM: multiple-reaction monitoring; MCPG: methylenecyclopropylglycine; S/N: signal-to-noise; UPLC-MS/MS: ultra high performance liquid chromatography-tandem mass spectrometry; %RE: percent relative error; %RSD: percent relative standard deviation.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have read and agreed to the published version of this manuscript.

Availability of data and materials

The datasets used during this work are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Weili Tao: Conceptualization, Methodology, Writing - original draft; Yu Yang: Data curation, Validation; Qiongjin Wang: Data curation, Software; Dong Chen: Funding acquisition, Investigation; Chuanyi Zhao: Investigation, Visualization; Qian Lv: Visualization, Validation; Yicun Chen: Writing – review & editing, Supervision

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Tables

Table 1

Accuracy (%RE) and precision (%RSD) data (n=6) for HGA in validation samples.

Analyte	Theoretical (µg/L)	Experimental (µg/L)	SD	Accuracy	Precision
HGA	10	9.98	0.14	-0.17%	1.37%
	150	150.03	1.98	0.02%	1.32%
	3500	3504.82	50.34	0.14%	1.44%

Table 2

Recovery data (n=6) of four litchi cultivars.

cultivars	Theoretical (µg/L)	Experimental (µg/L)	Recovery (%)	RSD (%)
Fengchuiliao	521.570	543.256	104.16	2.18
Fenghua	25.207	26.881	106.64	3.49
Gualv	2224.97	2190.514	98.45	3.03
Linglan	3512.46	2568.825	101.60	2.98

Table 3

Intra- and inter-day accuracy and precision data (n=6) for HGA in QC samples.

QC(µg/L)	Intra-day				Inter-day			
	Mean	SD	RE (%)	RSD (%)	Mean	SD	RE (%)	RSD (%)
7	7.02	0.06	0.29	0.91	7.04	0.12	0.61	1.74
15	15.01	0.12	0.07	0.79	15.09	0.14	0.57	0.91
100	103.07	1.55	3.07	1.51	102.58	1.66	2.58	1.62

Table 4

The UPLC-MS/MS method was applied to detect HGA contents in different parts (pericarp, pulp, seed, leaf and branch) of four litchi cultivars at two maturity stages, ANOVA analysis and Independent-Samples T Test were used to found the significant differences of HGA concentrations within various cultivars and between different maturity stages, respectively.

Cultivar	HGA (μg/g)										
	pericarps		Sig.	arils		Sig.	seeds		Sig.	leaves	
	maturity	immaturity		maturity	immaturity		maturity	immaturity			
Fengchuiliao	0.629±0.016	0.869±0.012	<0.001	15.155±0.621	25.231±0.247	<0.001	31.607±0.273	35.140±0.421	<0.001	1.532±0.027	2
Fenghua	0.053±0.002	0.042±0.002	0.003	1.002±0.013	0.334±0.008	<0.001	30.837±0.473	54.325±0.747	<0.001	0.512±0.004	11
Gualv	1.653±0.036	0.053±0.001	<0.001	0.330±0.005	16.076±0.161	<0.001	8.360±0.084	12.239±0.164	<0.001	0.929±0.008	5
Linglan	0.052±0.002	0.040±0.001	0.001	0.292±0.004	0.507±0.008	<0.001	0.116±0.001	15.092±0.217	<0.001	0.272±0.010	0
F-value	4307.822	14073.266		2178.683	30380.580		11219.190	3254.737		3895.737	
Sig.	<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.001	

Figures

Figure 1

Pericarps, arils, seeds, leaves and branches which were divided from four different Litchi cultivars in two maturity stages.

Figure 2

The fragmentation pattern of HGA and the product ion mass spectra of HGA with m/z of 142.10 at a CE of 11.0 V.

Supplementary Files

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