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DEVELOPMENT OF STACHYOSE QUANTITATIVE DETERMINATION IN *STACHYS AFFINIS* BUNGE BY LC - MS AND ITS APPLICATION FOR ESTABLISHMENT OF STACHYOSE REFERENCE STANDARD

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Summary

Development of Stachyose Quantitative Determination in *Stachys affinis* Bunge by LC - MS and its Application for Establishment of Stachyose Reference Standard

Stachyose is the main ingredient in *Stachys affinis* rhizomes and has many pharmacological effects such as lowering blood sugar, strengthening the immune system, and relieving constipation, etc. To monitor the quality of the new material and its products, the quantitative determination of stachyose in *Stachys affinis* Bunge rhizomes was developed according to the guidelines of ICH (2005) and AOAC (2012). Besides, the established procedure was applied to determine the purity of stachyose as following requirements of a reference standard.

The procedure was UPLC-MS system (ACQUITY QDa Waters); Phenomenex Gemini NX-C_{18} (4.6 × 150 mm; 5 µm) column; acid formic 0.05% and MeOH as mobile phase in isocratic mode; flow rate of 0.5 mL/min; detector MS/ESI- (665 *m/z*); cone voltage 40 V; capillary voltage 0.8 kV. Standards and test samples are all diluted in water before analysis.

Keywords: *Stachys affinis* Bunge, *Stachyose, Reference standard, LC-MS.*

1. Introduction

Vietnam is considered as a country with great potential for medicinal herbs in Southeast Asia and in the world. According to the Agency of Traditional Medicine Administration, out of a total of nearly 5,000 known medicinal plant and mushroom species, there are many species that have the potential to be exploited to create medicinal materials to serve market demand [1]. However, according to the report of the Agency of Traditional Medicine Administration, Vietnam can only supply 25% of the domestic demand for medicinal herbs, and most of the 75% depends on imports from mainly pharmaceuticals China. Therefore, the quality control of imported medicinal herbs is of great concern at present.

Stachyose ($C_{24}H_{42}O_{21}$) is a tetrasaccharide containing two α-D-galactose units, one α-D-glucose unit and one β-D-fructose unit linked as follows: *O*-α-D-galactose-(1→6)-*O*-α-galactose-

(1→6)-*O*-α-D-glucose-(1→2)-α-D-fructose (boiling point: 110°C, pKa: 11.63, pKb: - 3.7 and solubility in water: 411 g/L) [2]

The rhizomes of *Stachy affinis* are a rich source of stachyose with concentrations up to 26,3% by dry weight (accounting for 80 - 90% of total carbonhydrates) [3]. While legumes, known as the major source of stachyose, contain less than 10% stachyose by dry weight [4],[5]. It could be seen that *Stachy affinis* is a potential source of stachyose to produce dietary supplement. Besides, stachyose has not yet been in the list of Vietnam reference substances bank and the LC-MS procedure that we have previously studied only determined stachyose in the *Stachys affinis* rhizomes total extract [6]. Therefore, establishing a stachyose reference agent, and at the same time developing and validating the stachyose quantification process in *Stachys affinis* rhizomes are essential for contributing to assess the quality of the raw materials.

2. Materials and methods

Object: 1.16 g Stachyose was isolated form *Stachys affinis* rhizomes [7] and determined to be over 95.0% pure on the whole preparation (indentified by IR, LC-MS and qualified the content by LC-MS). *Stachys affinis* rhizomes provided by Tipharco Pharmaceutical Joint Stock Company (Ward 9, My Tho City, Tien Giang Province, Vietnam), then washed, dried at 60°C until the weight is constant. Grind to a fine powder using a sieve of 180/125, and store at room temperature.

Equipment: UPLC-MS system (ACQUITY Arc System - ACQUITY QDa Mass Detector; Waters); Phenomenex Gemini NX-C_{18} (4,6 × 150 mm; 5 µm) column; analytical scales Sartorius CP-225D (0.01 mg); ultrasonic tank DH-Elma T840; Labconco Protector Glove Box.

Chemicals: Acetonitrile, methanol and formic acid (HPLC grade; purchased from Merck), double distilled water (made in University of Medicine and Pharmacy HCMC) for LC-MS.

***Reference materials*:** stachyose hydrate isolated from *Stachys tuberifera* (Sigma-Aldrich), lot number: MFCD00149457, content: 98%, moisture content: 9%; fosfomycin disodium (Sigma-Aldrich), lot number: MFCD22741288, content calculated on anhydrous preparation: 99.0%, moisture content: 5.3%; D-lactose monohydrate (Sigma-Aldrich), lot number: MFCD00166994, content calculated on anhydrous preparation: 100.0%, moisture content: 5.3%; folic acid (DSM Human Nutrition & Health), lot number: UT19040080, content calculated on anhydrous preparations: 100.5%, moisture content: 7.5%; and some standard substances provided by The Institute of Drugs Quality Control - Ho Chi Minh city (IDQC - HCMC): ascorbic acid, lot number QT016100420, content calculated on the original preparations 99.9%; sodium valproate, lot number QT327 010618, content calculated on the original preparation 95.3%; clavulanate potassium, lot number QT204 060418, content calculated on the original preparation 41.7%.

3. Research method

Development of the quantitative process by UPLC-MS

Investigate the conditions of the mass spectrometer such as cone potential (20 - 25 - 30 - 35 - 40 - 50 V) and capillary potential (0.3 - 0.5 - 0.8 kV) to obtain the largest stachyose signal on the chromatogram. The procedure using ESI (-) mode with *m/z* number 665 to detect stachyose, was inherited from the previous work of the research team [5].

Investigate the chromatographic conditions and sample preparation conditions based on separation parameters of the sample (the signal of stachyose is the highest, its asymmetry is in range 0.8-2.0, its number of theoretical plates is greater than 2000): ratios of mobile phase (ACN-HCOOH x% or MeOH - HCOOH x%) in isocratic mode, internal standard (D- lactose monohydrate, folic acid, fosfomycin disodium, clavulanate potassium, sodium valproate, ascorbic acid), extraction solvent (water, MeOH), extraction temperature (ultrasonic extraction combined with temperature at RT - 60 - 80°C), ratio of extraction solvent (1:5, 1:10, 1:15, 1:20 w/v), extraction time (5 - 10 - 15 - 20 minutes) and number times of extractions.

The procedure was validated according to the guidelines of ICH (2005) [8] and AOAC (2012) [9] for systematic suitability, specificity, linearity, limit of detection, and limit of detection. quantification, repeatability, intermediate precision, and precision.

Establishment of stachyose reference standard: packing, evaluating the homogeneity among the vials after packaging according ISO guide 35:2017 [10], evaluating the quantitative results in relation to two GLP-certified laboratories including Department of Standards and Reference Substances establishment and Department of Metrology Physics (IDQC - HCMC) and determining the declared value and measurement uncertainty according to ISO 13528:2015 [11]. The LC-MS method developed and validated above was applicable for the purity quantification of the pure substance.

4. Result

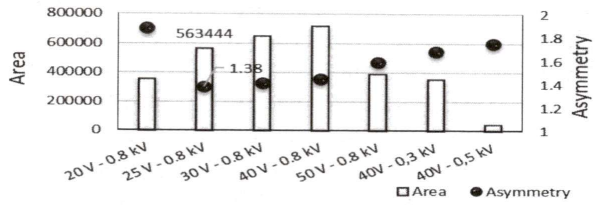


Fig. 1. Investigation results of the mass spectrometer conditions of the quantitative analytical procedure of stachyose in *Stachys affinis* rhizomes

Based on the obtained values, it was found that at low cone potential values, the peak area parameters were low, and asymmetric values were not in the required range 0.8-1.5 (Fig. 1). These parameters gradually optimized when the cone potential was increasing. As the cone potential reached to 50 V, the peak area decreased, the asymmetry coefficient was not complied. This could be explained by the strong bombardment process causing the radical ions quickly broken up into smaller fragments than *m/z* 665, leading the gradually lower *m/z* 665 signal. Therefore, the cone potential 40V and capillary potential 0.8 kV was chosen for MS condition of the procedure.

The results of applying different chromatographic conditions (Fig. 2) showed that, when the proportion of organic solvents (MeOH, ACN) in the mobile phase increased, the retention time of the stachyose peak was significantly improved and the peak area also increased. With the solvent ratio H_2O - MeOH (85:15), the peak area was high enough, and the tailing factor was still acceptable. When the formic acid concentration gradually increased from 0.025 % to 0.1%, the noise of the peak signal increased.

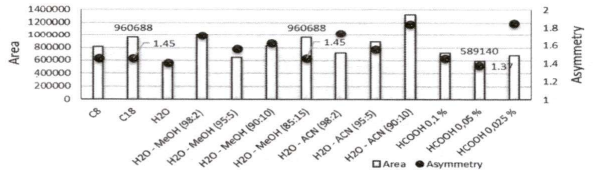


Fig. 2. Investigation results of the chromatographic conditions of the quantitative analytical procedure of stachyose in *Stachys affinis* rhizomes

Because of using ESI (-) mode, D-lactose monohydrate, folic acid, fosfomycin disodium, clavulanate potassium, sodium valproate, ascorbic acid compounds were chosen as internal standards for further study. In case of using folic acid, sodium valproate and lactose, their retention times were about 2 - 3 minutes with mobile phase systems requiring a high proportion of organic solvents. Thus, the procedure had to apply gradient mode for mobile phase which cost more analysing time. Concerning ascorbic acid, clavulanic acid and fosfomycin, when analysed under the same chromatographic conditions as stachyose, the retention times respectively: 5.195; 4.586 and 6.445 (minutes). However, the result of performing systematic compatibility of the procedure was not complied when using ascorbic acid (yielded a %RSD (n=6) of an $S_{Stachyose/ascorbic}$ acid ratio of 4.25%). Clavulanic acid is easily degraded under light to two absorption peaks. Therefore, fosfomycin was chosen as the internal standard.

Continuing with different conditions of the extraction method (Fig. 3), the results showed that using ultrasonic methods at RT, solvent: H_2O, ratio of 1:15 (weight of sample / volume of solvent) in 10 minutes and only one time extraction gave the highest efficiency (47.44%).

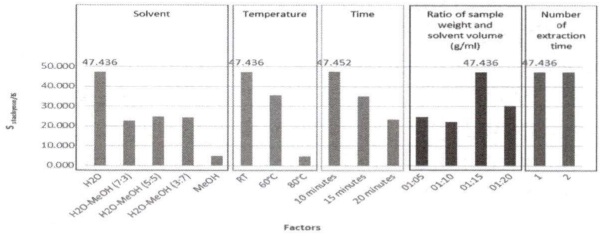


Fig. 3: The result of investigating extraction's conditions of the quantitative analytical procedure for stachyose in *Stachys affinis* rhizomes.

The final procedure for quantifying stachyose in *Stachys affinis* was developed as follows:

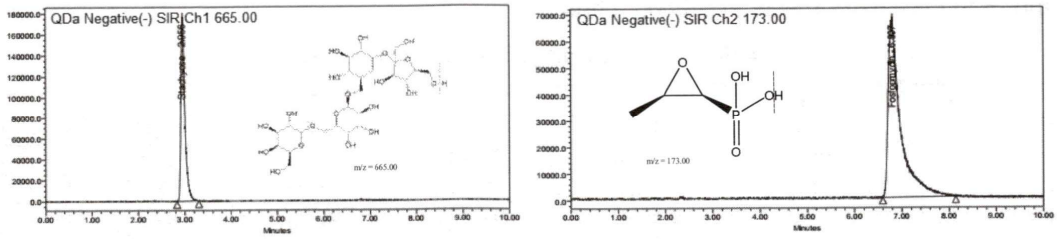
- Internal standard stock solution: The solution 1000 µg/ml fosfomycin in water.
- Stachyose standard stock solution: The solution 1000 µg/ml stachyose in water.
- Standard sample: Dilute the stock standard solution and the internal standard stock solution to obtain a mixture of 25 µg/ml stachyose and 25 µg/ml fosfomycin. Filter through a 0.22 µm filter before analysis on LC-MS.
- Test sample: Disperse 100.0 mg of test sample in 1.5 ml of distilled water, and sonicate for 10 min. Then centrifuged in 5 minutes with 5000 r/m, dilute 100 µl of supernatant to 10 ml by distilled water (Solution A). Take 400 µl of solution A and 250 µl of the internal standard stock solution into a 10 ml volumetric flask, add distilled water to make 10 ml. Filter through a 0.22 µm filter before analysis on LC-MS.
- UPLC-MS conditions: Phenomenex Gemini NX-C_{18} column (4.6 × 150 mm; 5 µm); Mobile phase: MeOH - HCOOH 0.05% in water (15:85); Injection volume: 5 µl; flow rate: 0.5 mL/min;

MS/ESI (-) detector (SIR mode: 665 *m/z* for stachyose, 173 *m/z* for fosfomycin internal standard); Cone potential: 40 V; Capillary potential: 0.8 kV.

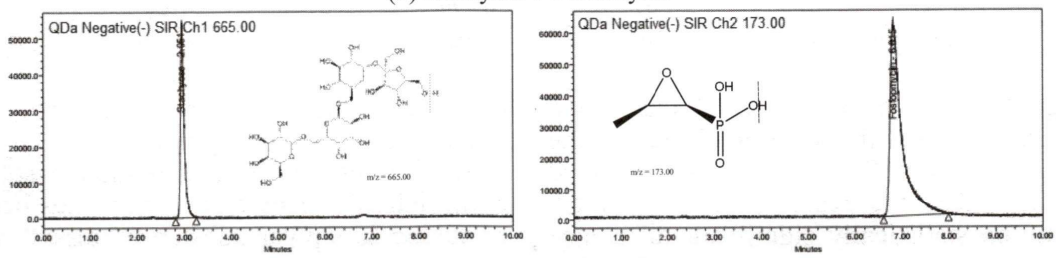
The quantification process was satisfactory in the direction of ICH and AOAC guidelines (Table 3).

Table 2. Result of specificity of the quantification process of stachyose in *Stachys affinis* rhizomes

	Stachyose		Fosfomycin		$T_{R\ Stachyose/IS}$	$S_{Stachyose/IS}$
	T_R (min)	S (IC×min)	T_R (min)	S (IC×min)		
Blank	-	-	-	-	0	0
Fosfomycin	-	-	6.68	6,563,214	-	-
Stachyose	2.95	1,030,907	-	-	-	-
Sample	2.951	278,514	-	-	-	-
Stachyose+fosfomycin	2.956	1,030,128	6.807	1,217,047	0.434	0.846
Sample+fosfomycin	2.951	297,729	6.815	1,110,279	0.433	0.268
Sample+stachyose+fosfomycin	2.951	1,258,223	6.826	1,149,419	0.433	1.095



(1) Stachyose + Fosfomycin



(1) Sample + Fosfomycin

Fig. 4. The LC-MS chromatogram of the quantification process of stachyose in *Stachys affinis* rhizomes

Table 3. Validation the quantification process of stachyose in *Stachy affinis* rhizomes

Criterion	Requirement	Result
System compatibility	The RSD of the retention time and the peak area of the principal peak to be analyzed in the standard and test samples are both ≤ 2 %; tail drag coefficient (T) is in the range of 0.8-2.0; apparent theoretical disk number (≥ 2000).	RSD_{tR}= 0.51%; RSD_{Spic}= 1.08%; T= 1.43; N_{bk}= 6305
Specificity	Mass spectrometry corresponds to standard samples and has been shown to be unaffected by excipients with respect to retention time and the software's peak purity check feature. The mass spectra of the peak in the sample and in the standard chromatogram were the same.	Complied (Fig. 4, Table 2)
Linearity range	Use regression analysis with t-test to check the significance of coefficients in the regression equation and F-test to check the appropriateness of the regression equation. R > 0,98.	$\hat{y}$ = 0.0338x; R^2 = 0.9988 Range: 0.94 - 56.40 µg/mL
Accuracy	The recovery range in 3 levels 80, 100, 120 % is 98-102 %. RSD ≤ 2 %.	Recovery (Confidence Level 95,0%): 100.70 ± 1.01%; RSD% (n=9): 1.31%
Repeatability	RSD ≤ 2 %.	Mean of stachyose content: 46.71%. RSD% (n=6): 1.06%.
Intermediate Precision	The results obtained should not be significantly different (using F-test with 95 % confidence interval to test). RSD ≤ 2 %.	Mean of stachyose content: 46.85%; RSD% (n=12): 1.18%
LOD -LOQ	Determine the minimum concentration at which the analyte response can be read.	LOD = 0.05 µg/mL LOD = 0.10 µg/mL

Establish stachyose reference substances

Stachyose is packed in brown glass vial, stopper with teflon buffer layer, N_2 inert atmosphere 99.99 %, relative humidity 10 %, stored at 2 - 8°C. 2.0 g of stachyose were packed in 100 vials of 20 mg each. The results of system compatibility determination of stachyose purity by UPLC-QDA in 2 laboratories were satisfactory with RSD% (n=6) of all chromatography parameters are less than 2.00% (resolution ≥ 3.0, theoretical plates ≥ 2000, symmetry ≥ 0.8 and ≤ 2.0). The relative repeatability of stachyose purity between the vials during the packaging process was less than 1,00% (the average purity value (n=12) was 97.22% and RSD% (n=12) was 0.98%). Thus, the packaging conditions are stable and consistent, and the vials are homogenized during the packaging process.

Table 3. Interlaboratory vial homogeneity assessment

Number	Purity (%)	
	LAB 1	LAB 2
1	98.30	98.45
2	96.87	97.55
3	96.15	95.46
4	97.07	96.59
5	96.76	97.14
6	97.70	98.62
Average	97.14	97.30
RSD %	0.78	1.22

Table 4. Robust A analysis results of stachyose

Cycle	0	1	2	3	5	6	7
=1.5s*	-	1.43	1.43	1.43	1.43	1.43	1.43
x* -	-	95.80	95.80	95.80	95.80	95.80	-
x* +	-	98.65	98.65	98.65	98.65	98.65	-
Average	97.22	97.22	97.22	97.22	97.22	97.22	97.22
SD	0.95	0.84	0.84	0.84	0.84	0.84	0.84
x*	97.11	97.22	97.22	97.22	97.22	97.22	97.22
s*	0.82	0.95	0.95	0.95	0.95	0.95	0.95

Using ANOVA analysis by Excel 2016 software to evaluate the data in Table 3, the results $F_{tn} = 0.08 < F_{0.05} = 4.96$, the obtained results show that the results are determined. There was no statistically significant difference in the purity of stachyose between the 2 laboratories. Thus, the chromatographic purity is independent of the laboratory participating in the evaluation.

According to the guidelines of ISO 13528, from the robust A analysis of the stachyose reference after 7 cycles (Table 4), the s* value did not change. The assigned value x*= 97.22%, the true standard deviation of the assigned value s* = 0.17. After excluding the values with Z - score >2, the published value is calculated from the remaining values (97.07%; 97.55%; 97.14%). The declared value of the stachyose reference material of chromatographic purity is 97.3% calculated on the preparation as-is. The measurement uncertainty is 0.123 calculated by the formula $\mu=(1.25\times s^*)/\sqrt{n}$. Thus, stachyose is eligible to register for the national standard with the determined content of 97.3% according to the status quo. Proceed to make a standard substance profile, label the standard vial, store at 0-8°C, protected from light.

5. Discussion

Stachys affinis rhizomes were selected as the subject of the study because the rhizomes contained the highest stachyose content. Stachyose is a very polar tetrasaccharide, so water was chosen as the extraction solvent for both high efficiency and cost savings because of removing less-polar substances dissolved in the sample.

The stachyose structure consists of two α-D-galactose units, a α-D-glucose unit, and a *β*-D-fructose unit without a chromogenic group. Conventional analysis encounters many difficulties. Most of the research and quantitative analysis of oligosaccharides in the world use specialized analytical columns for carbohydrates such as Aminopropyl AP, amine [12], the corresponding column USP L17[5] or the fourth-generation column C_{18} BEH HILIC [13] and the combination of probes with sensitive and specific properties such as: RI [14],[15], MS [16],[17]. The types of reversed phase columns C_8 and C_{18} have not been identified yet any application for this compound. Currently, column C_{18} is often available at research institutions and drug laboratories in Vietnam, so the procedure will have a higher applicability, and this is also a new contribution of the research.

In this study, the content of stachyose hydrate from Sigma-Aldrich was published 98.0% with high humidity (9.0%) and stachyose reference standard is not available in the list of Vietnam standard bank. The established one was higher

purity and could be the good replacement in Vietnam as an analytical standard in identifying the components of various plant extracts.

6. Conclusion

The procedure for the quantification of stachyose in *Stachys affinis* rhizomes by LC-MS was validated and met the requirements of the ICH and AOAC guidelines so that it can be applied to quality control of the input materials. Besides, this method was applied for determination of the content of the standard according to the standard setting procedure from which the assigned value is given. Stachyose has been established as a reference material with a purity of 97.3% calculated on the inoculant as-is, which meets the requirements of a standard used in the quality control of stachyose in raw materials and products.

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MICROSCOPIC CHARACTERISTICS, ANTIOXIDANT ACTIVITIES, AND CHEMICAL COMPOSITIONS OF *OCHNA INTEGERRIMA* LEAVES AND STEMS

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Summary

Microscopic Characteristics, Antioxidant Activities, and Chemical Compositions of *Ochna integerrima* Leaves and Stems

Ochna integerrima is a typical and precious plant for spring in Vietnam. The plant has been used traditionally for treatments of various ailments. The microscopic transverse section of its leaf and stem reveals the presence of phloem arcs from the leaf midrib. The upper and lower arcs are continuous and separated. The sclerenchyma contains two rings with a continuous outer and an interrupted inner in the stem. The MeOH extracts of the leaf and stem possess significant total phenolic