MINISTRY OF EDUCATION AND TRAINING

INSTITUTE OF INDUSTRIAL CHEMISTRY OF VIETNAM

DO THANH HA

RESEARCH OF THE TECHNOLOGY TO SEPERATE CATECHINS FROM THE GREEN TEA (*CAMELLIA SINENSIS* L.), TRANSFORMATION TO CREAT THE DERIVATIVE O-ACETYL CATECHIN AND INVESTIGATE THEIR FREE RADICAL SCAVENGING ACTIVITY

Speciality: Organic chemistry

Code: 62.44.01.14

SUMMARY OF PhD THESIS IN CHEMISTRY

HANOI - 2017

1. Meaning and purpose of the thesis

Tea is used as a beverage and a remedy in the oriental medicine from thousands years of BC. However, the promotion of research into the mechanism and the application of the phenolic active ingredients in the green tea to modern the medicine began only in the second half of the 20th century. Until now, with the strong antioxidant activity, catechins in the green tea (up to 30% dry content) have been used in the cosmetics and the functional foods. In medicine, catechins have been used in the treatment of many types of the cardiovascular disease, inflammation and cancer. These active ingredients are especially effective when used in combination with other medicines that treat the inflammatory, allergic, cardiovascular diseases such as Alzheimer's, obesity, insulin-dependent diabetes, Parkinsons's syndrome, HIV-1, skin cancer and some other types of cancer.

In the year 2006, the US Food and Drug Administration (FDA) recognized the extract from the green tea, polyphenols from the green tea and the catechins are all types of the pharmaceuticals and the functional foods authorized for use in the United States. In the leaves of the green tea, the major component of catechins is epigallocatechin gallate (EGCG, containing> 50% of total catechins) is the most active. Currently, the pharmaceutical companies around the world have separated EGCG to register and commercialize as a single agent. In Vietnam market, there have also appeared many cosmetic products, functional foods and pharmaceuticals containing the green tea polyphenols and EGCG imported from China.

Since the year 2000, the local research institutions such as Military Medical Institute, Institute of Chemistry under Vietnam Academy of Science and Technology, TRAPHACO Company, Mediplantex Company, Hanoi University of Medicine, Hanoi University of Pharmacy, Ho Chi Minh City University of Medicine and Pharmacy have started to extract the total polyphenols from the green tea and began to study the isolation of catechins from the green tea as well as the phenolic compounds from other sources with the aim to apply in the pharmaceutical and cosmetic field. These researches have

gained the initial successes in the field of the basic chemistry and the biological activity of catechins from the green tea, which is a prerequisite for the future research and for the future technological development.

The purpose of this thesis is " ***Research on the technology to separate catechins from the green tea (Camellia sinensis L.), transformation to creat the derevative O-acetyl catechins and to investigate their free radical scavenging activity***". This is a new research direction in Vietnam, having a very promising application in the pharmaceutical industry.

2. Subjects and aim of the thesis

The research object of the thesis is the green tea (Camellia sinensis (L.) O.Kuntze.), the technological system to extract catechins from the green tea and catechins extracted from the green tea.

Main aim of the thesis:

- From the green tea (Camellia sinensis L.) to prepare (extracte and semi-synthesize) the end-active ingredients including catechins and the acylated derivatives;

- Analysis and evaluation of the chemical structure and the biological activity of the selected active substances from the above substances.

3. New contributions of the thesis

3. 1. The thesis has been successful in applying the advanced liquid extraction/solid continuous back flow technique using water as a solvent with the support of the citric acid to isolate catechins from the green tea (Camellia sinensis L.) in pillot scale of 100 kg the green tea/batch. The extraction technology gave the high extract efficiency of 12.5%. Catechins contain mainly EC, EGCG, EGC, ECG > 90%, in which EGCG> 50%. This technique is well suitable for use in the production of the pharmaceutical organic substances and the functional foods. This is the first time in Vietnam that the continuious back flow liquid/solid extraction technique of catechins from the green tea has been carried out on a semi-industrial level.

3. 2. The extraction and the refinering technique of catechins from the total catechins using the high performance liquid chromatography technique using Diaion HP20 SS conjugate separation column connected online with gel filtration column high performance Sephadex LH20 with the short chromatographic separation times, has been established. The separation efficiency reached ~ 80%, major EC, EGC and EGCG catechin with a purity of ≥ 95% can be used for the pharmaceutical use. For the first time in Vietnam, the high performance liquid chromatography technique using the conjugate coupling system was applied to seperate catechins in the semi-industrial level.

3. 3 EGCG purification by column chromatography on the conventional phase of silica gel by the direct phase chromatography was performed in the chromatography process running by the chromatography solvent diluted with the citric acid, this is a new technological contribution for the practical application of chromatographic separation on the semi-industrial level to get the catechins in Vietnam with the reducing costs. For the first time, the purification of EGCG by column chromatography on silica gel has been studied. The technology has been used in the pillot level which 100 times of seperation. After 100 times of seperation the process has been still effective.

3. 4. O-acetyl derivatives of EC, EGC and EGCG catechins were semi - synthesized. The ability to remove the free radicals of catechins from the green tea and acetate derivatives in comparison with resveratrol has been evaluated. Acetyl derivatives have a reduced activity against the original catechins but remain high: EC O-acetyl; ECG O-acetyl and EGCG O-acetyl have an EC50 value of 45.64 μg/ ml respectively; 52.99 μg/ ml and 34.58 μg/ ml. The decrease of the free radical scavenging activity of the O-acetyl catechins increases the stability of these compounds.

4. Structure of the thesis

The thesis consists of 163 pages with 18 tables of data and 50 images, diagrams. The structure of the thesis includes: Introduction (2 pages); Document overview (27 pages); Experiment (25 pages); Results and discussion (46 pages); Conclusion (2 pages); New contribution (2 pages); References (8 pages, 76 references); List of works published

by the author. There is also an annex detailing the NMR spectrum of the active substances studied in the thesis.

II. CONTENT OF THE THESIS

INTRODUCTION

The introduction refers to the scientific meaning, practicality, objectives and research tasks of the thesis.

CHAPTER 1: OVEERVIEW

+ Overview of the green tea, the chemical composition of tea products;

+ Catechins composition, biological activity and applied research in the cosmetics, the pharmaceuticals of EGCG and the applications in the cosmetics, the pharmaceuticals of EGCG and catechins from the green tea;

+ An overview of the technological studies on the extraction and preparation of catechins and their derivatives.

CHAPTER 2: EXPERIMENT

2.1. Research subjects

***A/ Green tea*:**

Green tea leaves - Camellia sinensis (L.) O.Kuntze was purchased from the households producing and trading tea in Dai Tu district, Thai Nguyen province, chopped tea is dried at 110^{o} C for 15 minutes to kill yeast, then re-homogenised to the average humidity of 5 - 10 %; grind to a size below the 1.5 mm sieve; put into closed HDPE bag, vacuum, store in dark at the temperature $<25^{o}$ C.

***B/ Total catechins from the green tea*:**

Total catechins are extracted from the green tea during the perfomance of the thesis, catechin has a light yellow color, total phenolic content is > 90% by HPLC. The concentration of EGCG in catechins is at least 50%.

***C/ Catechins from the green tea*:**

Catechins in the green tea were isolated from the total catechins during the performance of the thesis, including: (-) - epicatechin; (-) - epigallocatechin; (-) - epigallocatechin-3-O-gallate.

2.2. Research method

2.2.1. Chemicals, instruments and equipments

This content was mentioned in Chapter 2, Section 2.2.1, page 32 of this thesis. This mentioned about the basic chemicals and laboratory equipments and instruments used in the research.

2.2.2. Method to extract the green tea

2.2.2.1. The method of the extracting reflux using alcohol

This is a method of the extraction of polyphenosl in the green tea that has been used in China and in the world, this method can be summarized as follows:

Tea flour is refluxed on a 20 liter Soxhlet with ethanol extraction in a suitable time. The extract is cooled, filtered and dried at the low pressure to remove the ethanol.

Next, the extact from the green tea has been liquid-liquid extracted with n-hexane to remove the les polarized substances. The aqueous extract has been liquid-liquid extracted with ethyl acetate to obtain catechins. Caffeine is removed from the total polyphenol by refluxing with chloroform over the suitable time at an appropriate rate. The powder after extraction of the remaining caffeine is called total catechins.

2.2.2.2. Continuous extraction method using water as a solvent

In the continuous extraction system, the wet tea powder through the grinding gear of the device is finely grinded to form a smooth paste. Then the material and solvent are given to the opposite sides of the extractor. In this process, the extracting material (usually under the smooth slurry) is moved upwardly along the tube extractor, where the material is in contact with the extraction solvent in the opposite direction. The soluble

substance in the raw material is always exposed to the extract containing lower solubility, the difference in the concentration of two liquid solid phase promotes the diffusion from solid to liquid is always occurring. The raw materials move far away, the extract concentration is becoming higher. After the concentration of the material in the fluid and the rate of translation reached the optimal level, the extraction may be finished, the concentrated extract will flow to one end of the extraction tube while the residue (almost no solvent) was taken off at one another end.

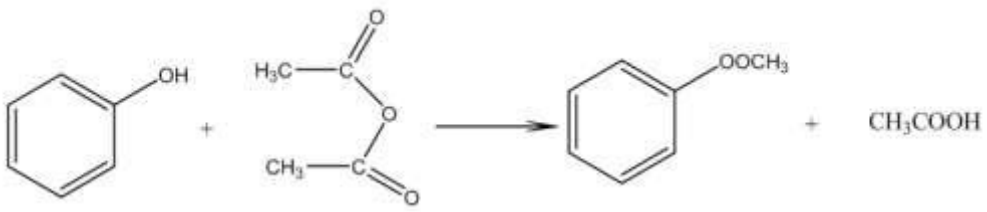
2.2.3. Methods of catechins separation

Catechins and EGCG were studied and optimized for the separation technology on semi-industrial high-pressure liquid chromatography systems with the diagonal separation columns of HP 20 connected to Sephadex LH 20 column of Center of the Pharmaceutical Chemistry under Institute of Industrial Chemistry of Vietnam.

2.2.4. Catechin acylation method

Acylation is the process to replace H atom in the -OH, -NH2 ... functional group by the R-CO- group. It needs not only the strong bases, but it also requires a reaction substance having a proton.

Acylated reaction with acetic anhydride follows the mechanism as below:



Acetic anhydride is essentially more stronger than carboxylic acid, so that the acylating reaction is stronger than carboxylic and a non-recovery phenolic reaction.

2.2.5. Methods of the analysis of the products

Thin layer chromatography (SKLM):

Thin layer chromatography is performed on thin silica gen with the appropriate

solvent systems. UV/VIS instructions 360 nm and current drug Von's were used as an indicator.

High Performance Liquid Chromatography connected to Mass Spectroscopy:

Catechins isolated from the green tea in Vietnam have been quantitatively and qualitatively analyzed by HPLC-MS on LC/MSD-Trap-SL equipment Agilen 1100; Institute of Chemistry (Vietnamese Academy of Science and Technology).

Nuclear Magnetic Resonance Spectrum:

EGCG, other products from EC, EGC, caffeine and derivatives were confirmed by 1H-NMR and 13C NMR spectra on the Brucke AVAN 500 MHz NMR machine of the Institute of Chemistry (Vietnamese Academy of Science and Technology).

2.2.6. Investigation design of the thesis

The objective of the study was to isolate catechins from Vietnamese green tea (Camellia sinensis L.), transformation to create the derivatives and initially to investigate some biological activities of some products. Investigation design has been set up as follows:

- To use the extraction methods using water and solvents (ethanol, methanol, chloroform, etc.) such as Soxhlet extraction, continuous back flow liquid/solid extraction and extraction liquid-liquid extracted to obtain phenolic compounds (containing catechins);

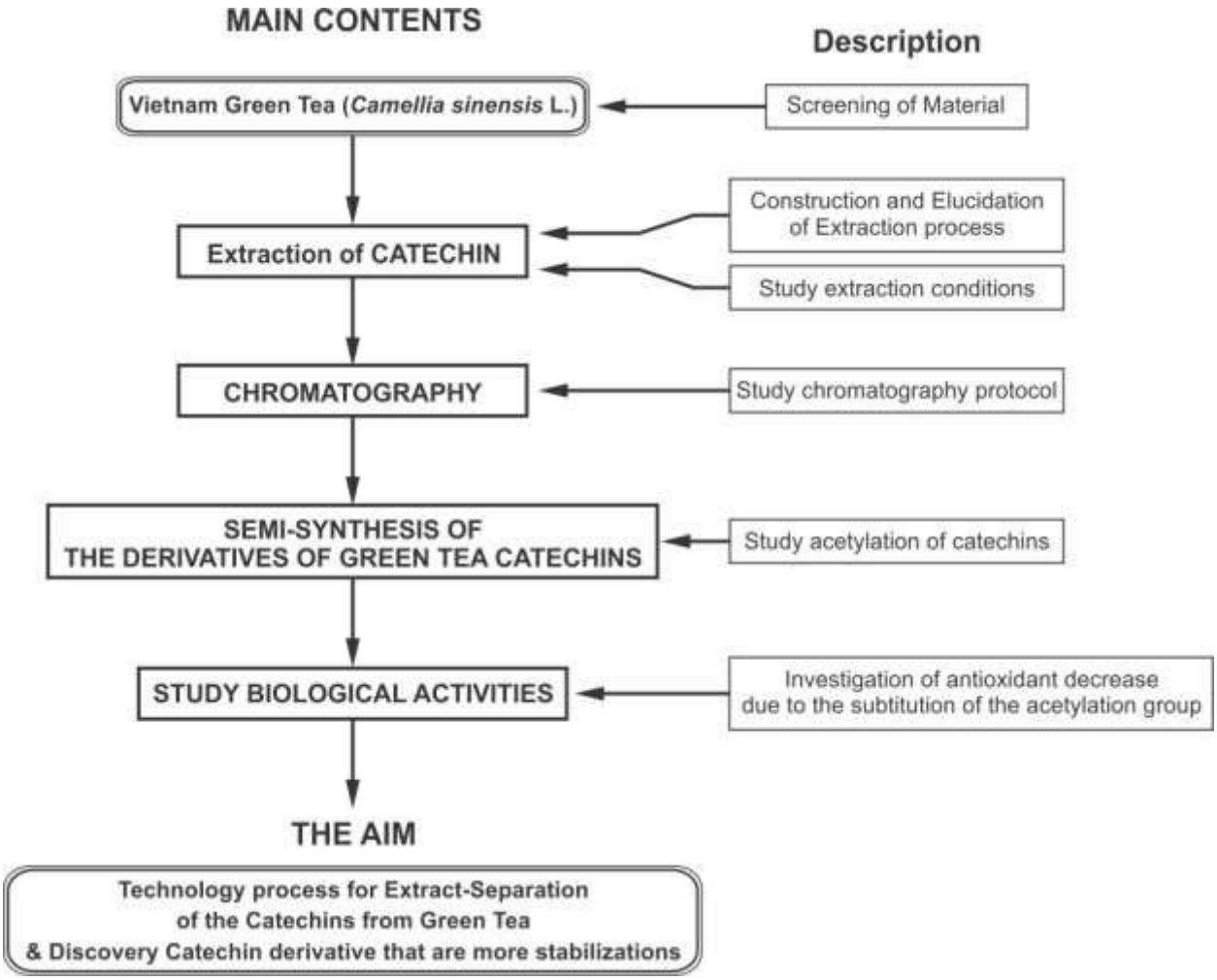
- To use the chromatographic methods (high performance liquid chromatography, liquid chromatography, thin layer chromatography, column chromatography) to seperate and purify catechins obtained;

- To use the method of the structural analysis by spectral types (infrared spectra, UV spectra, nuclear magnetic resonance spectra, mass spectra) to determine the chemical structure of the pure catechins obtained;

- To investigate the semi-synthesis of acetyl derivatives of catechins to find more

stable derivatives under the normal storage conditions with the aim to use as the antioxidant preservatives;

- To carry out in vitro studies to test and evaluate the antioxidant activity of catechins and their derivatives.



2.3. Experiment

This section is mentioned in Chapter 2, from page 30 to page 54. Detailed description of the experiments during the performance of the thesis with the aim to investigate the extraction conditions, the semi-synthesis of catechins in the green tea, the semi-synthesis of catechins and investigation of their biological activity.

CHAPTER 3: RESULT AND DISCUSSION

3.1. Investigation to set up the process extracting the total catechins from the green tea

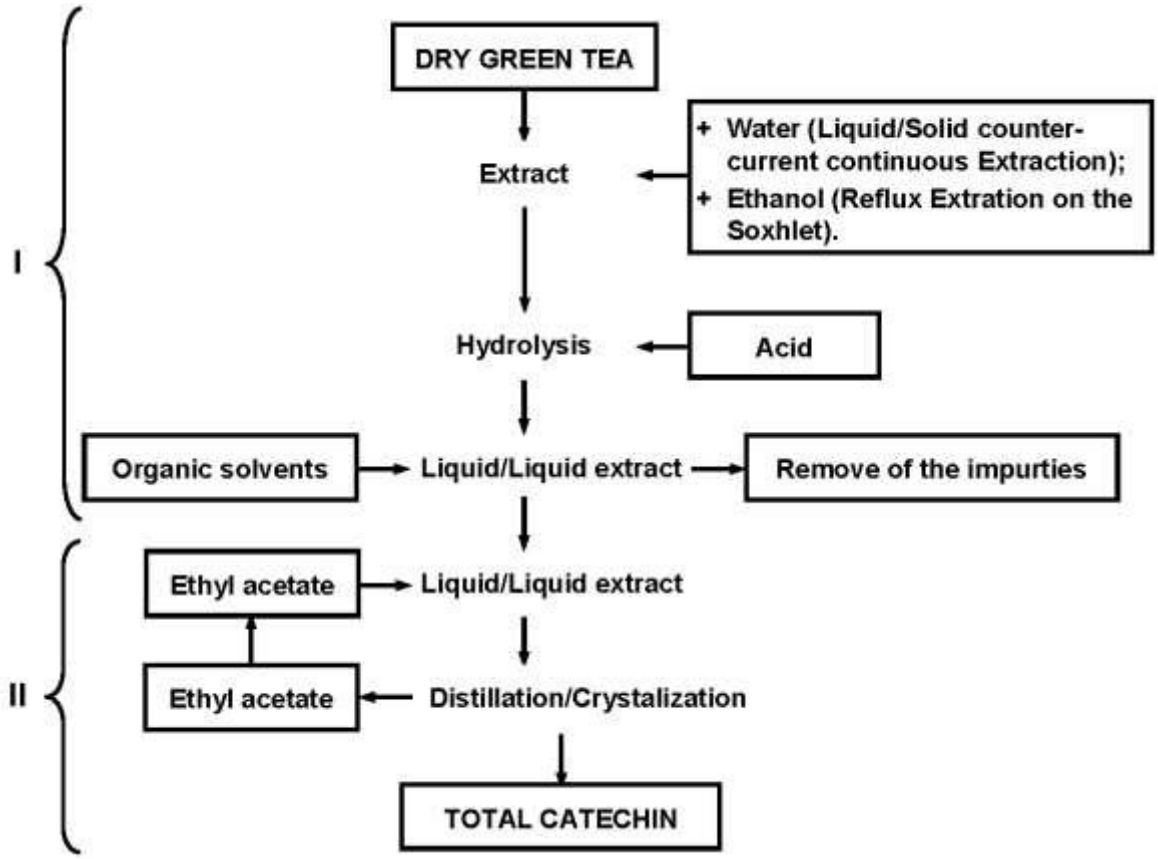
3.1.1. Invstigation of the general technological process to extract the total catechins from the green tea

To set up and to survey the extraction technologies of catechins in the green tea according to the process applied in the world.

According to this process, catechins are weakly polar, easily soluble in water and the polar organic solvents such as ethanol, methanol:

+ Extraction used ethanol: The material is extracted with ethanol on Soxhlet extraction system, then the extracted mixture is dried at low pressure to remove ethanol and to get extract;

+ Extraction used water: The material is given to the continuous back flow liquid/ solid extraction system with the citric acid solution (acid content ~ 0.05% compared to the material), the speed of back running of solvent/material is adjusted appropriately.



Polyphenols in ethanol extract or warter extract are then hydrolyzed with 1% HCl and then catechins have been extracted from the tea leaves by liquid-liquid extraction with the appropriate organic solvents such as n-hexane, chloroform and ethyl acetate respectively to remove chlorophyll and caffeine firstly, then extract to get the catechin.

3.1.2. Investigation on the extraction of the total catechins by ethanol according to Soxhlet method

The extraction of the green tea with ethanol according to Soxhlet method can be get a total catechins having a light yellow color, from 5 kg of tea material can be obtained 470 g total catechins, this is the acceptable rate for the method of reflux extraction. This result is equivalent to the previous research results in the world.

The composition and relative content of the catechins were analyzed by HPLC/MS.

The content of some catechins in the total catechins extracted by ethanol

No	Retention time (min)	MS	Concentration (%)	Substance
1	9,874	458	53,67	EGCG
2	10,309	290	16,86	EC
3	10,683	306	13,12	EGC
4	11,735	442	16,24	ECG

3.1.3. Investigation on the green tea extracted by the continuous back flow method

Survey and setting up the extraction process of tea by water on the continuous liquid/solid extraction systems has reduced the extraction time by 4 hours per 100 kg of dry tea material, reducing the cost of using n-hexane and ethanol, the result as follow:

+ Totally from 100 kg of dried tea material can be obtained 12.5 kg of total catechins, reaching an extraction efficiency of approximately 12.5%.

+ The composition and relative content of the catechins have been analyzed by HPLC/MS.

Composition of the total catechin extracted by the continuous back flow method

No	Retention time (min)	MS	Concentration (%)	Substance
1	9.18	290	15.46	EC
2	11.8	458	50.62	EGCG
3	21.19	306	29.24	EGC
4	24.02	442	2.62	ECG

So that in terms of the technology deployment, the back flow extration by water has

a greater advantage relating the time and the solvent usage. The recovery of caffeine and chlorophyll byproducts is easier because in this mixture there is not present of fat impurities and oil compared to the extraction by ethanol.

3.2. Investigation on the separation and purification of catechins and EGCG from the total catechins of the green tea

3.2.1. Investigation on the selection of the adsorbent that can be reusable as a static phase to seperate from total the Catechins in the green tea

The chromatographic separation methods having the great different characters have been used to separate EGCG from catechins in the green tea.

The condition and equipment are as follow:

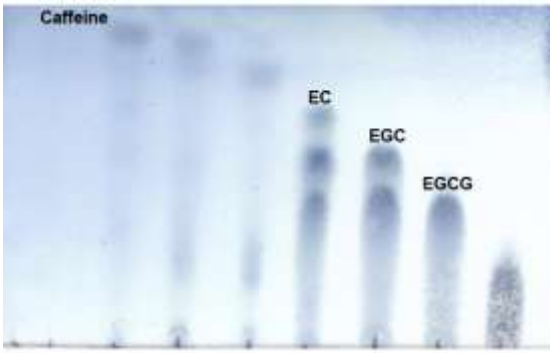
1 - Silica gel column modified by the mobile chromatography phase.

2 - RP/NP dual phase silica gel;

3 - Sephadex LH 20;

4 - Pore polymer material has the large molecular sieve HP 20 SS.

The chromatograms give the similar approximate results. EGCG will be the last of 3 major catechins of the green tea (EC, EGC and EGCG) eluated from the column. The thin-layer chromatography (TLC) diagram below show the most characteristic result for the chromatographic seperation of EGCG.



The phenomenon of catechins eluted from the chromatographic column in the similar order on the various static chromatographic phases was explained by the strongly adsorbion of catechins on the static phase surface. Accordingly, catechins will be eluted

in the order of solubility of each in the solvent mixture.

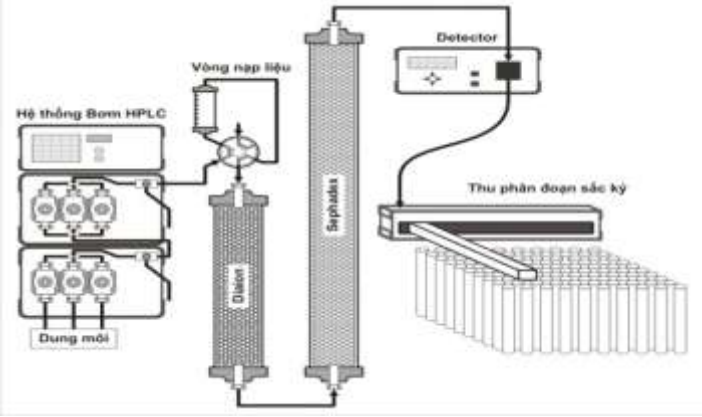
From the results of the investigation on the separation chromatographic efficiency of EGCG on the various static phase mixtures, combined with the reference and improvement of the catechin separation chromatography is similar to the methods widely used on the world. We have developed a technological method "Isolation of catechins from the green tea by the high performance liquid chromatography using Diaion HP20 SS and Sephadex LH20 chromatographic columns with the mobile phase solvent/water."

3.2.2. Investigation and testing of EGCG separation technology on the high liquid chromatography MP/HPLC pillot

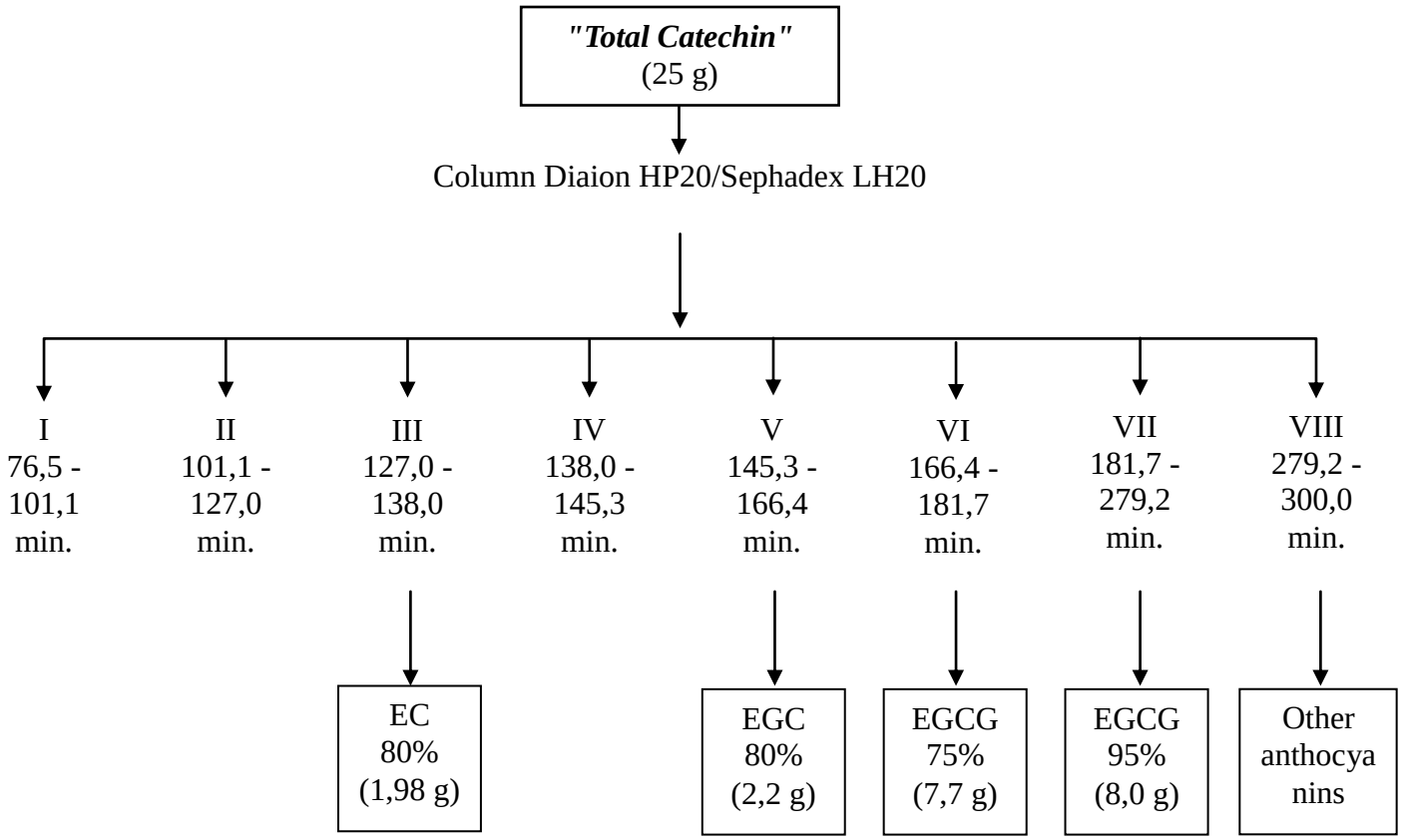
Silica gel 60 Merck (15 - 40 μm) modified by the mobile chromatography phase is the best option for the chromatographic separation to obtain EGCG at medium and small pillot scale (<100 kg/year), the factors affecting the total catechin in the green tea on MP/HPLC Silica gel 60 chromatography column have been investigated to set up the production process of EGCG.

3.2.3. Investigation on the seperation of catechins by HPLC on Diaion HP20 and Sephadex LH20

Diaion HP20 SS is a resin used as a carrier for the cationic or anionic ion exchanger, this resin itself is capable of exchanging anion and cation. When cleansing the catechins in alcohol/water through the chromatography column loaded this carrier, the difference in the adsorption/adsorpted affinity of the catechins in the order of the acid separation of the catechins in the solvent. From there, the seperation efficiency of the catechins will be enhanced as the mixture is further eluted through Sephadex LH20 gel column.



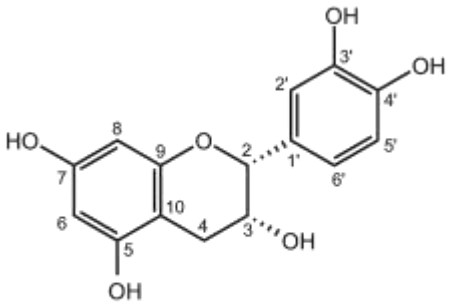
From 25 g total catechins, 8 fractions were obtained in which sections III, V, VI and VII contained the major catechins of the green tea. Segments containing EC, EGC and EGCG are excluded totally solvent at 55^{o} C, at the pressure 80 mbar; These substances are purified by recrystallization in a suitable solvent.



Schematic diagram of catechins in the green tea: EC; EGC; EGCG

3.2.3.1. Evaluation of segment III

Segment III includes the fractions from 127 → 138 minutes, containing the major component epi-catechin (EC; Rf ~ 0.56) with a content of> 80% (LC/MS); recrystallized in the ethanol yielded 1.98 g of epi-catechin needle-shaped crystal, white; The ESI-MS spectrum m/z = 290.5 corresponds to the molecular weight 290; molecular formular $C_{15}H_{14}O_6$; Melting point: 242^{o} C.



***epi*-Catechin**
(2*R*,3*R*)-2-(3,4-dihydroxyphenyl)chroman-3,5,7-triol

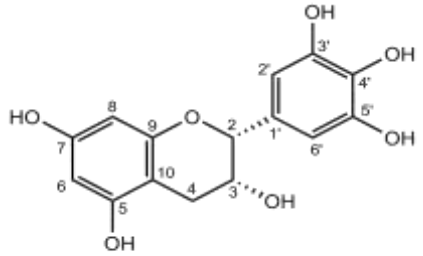
Chemical shifts of epi-catechin

No	**δ_H (ppm)**	**δ_C (ppm)**
2	4,74 *brs*	78,07 *d*
3	4,01 *mbr*	64,95 *d*
4	Ha: 2,49 *m* (overlap DMSO-d_6) Hb: 2,68 *dd J* = 4,5 Hz; 16 Hz	28,19 *t*
5	- - -	156,52 *s*
6	5,89 *d J* = 2,5 Hz	95,13 *d*
7	- - -	156, 24 *s*
8	5,72 *d J* = 2,5 Hz	94,13 *d*
9	- - -	155,77 *s*
10	- - -	98,53 *s*
1’	- - -	130,63 *s*
2’	6,89 *d J* = 1 Hz	114,90 *d*
3’	- - -	144,44 *s*
4’	- - -	144.51 *s*
5’	6,66 *mbr*	114,78 *d*
6’	6,65 *t J* = 3 Hz	117,97 *d*

Solvent DMSO-d6

3.2.3.2. Evaluation of the segment V

Segment V includes the fractions from 145.3 → 166.4 minutes, the main component is epi-gallocatechin (EGC; Rf ~ 0.4), content of 75%; This compound is difficult to crystallize in the alcohol and water; Purified on silica gel (FC) to obtain 2.2 g of clean EGC; The ESI-MS spectrum m/z = 306.7 corresponds to the molecular weight = 306; chemical formular $C_{15}H_{14}O_7$; Melting point: 276^o C.



***epi*-Gallocatechin**
(2*R*,3*R*)-2-(3,4,5-trihydroxyphenyl)chroman-3,5,7-triol

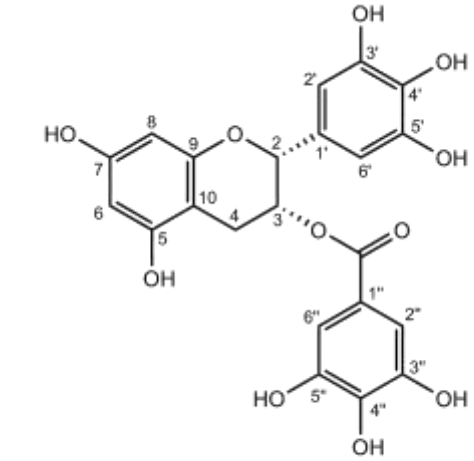
Chemical shifts of epi-Gallocatechin

Stt	**δ_H (ppm)**	**δ_C (ppm)**
2	4,66 *brs*	78,38 *d*
3	3,99 *d J* = 2 Hz	65,27 *d*
4	2,47 *dd J* = 7 Hz, 16,5 Hz 2,67 *dd J* = 5 Hz, 16,5 Hz	28,04 *t*
5	9,11 *brs* (Ar-O<u>H</u>, C5)	156,44 *s*
6	5,88 *d J* = 2 Hz	95,41 *d*
7	8,92 *brs* (Ar-O<u>H</u>, C5)	156,77 *d*
8	5,72 *d J* = 2 Hz	94,44 *d*
9	- - -	155,99 *s*
10	- - -	98,95 *s*
1’	- - -	130,06 *s*
2’ & 6’	6,38 *brs* (2 H)	106,36 *d* (2 C)
3’ & 5’	8,69 *brs* (2 Ar-O<u>H</u>, C3’ & C5’)	145,61 *s* (2 C)
4’	7,92 *brs* (Ar-O<u>H</u>, C4’)	132,39 *s*

Solvent DMSO-d6

3.2.3.3. Evaluation of segment VI and VII

Segment VI and VII include the fraction 166.4 → 181.1 minutes and 181.1 → 279.1 minutes with the main constituent epi-gallocatechin gallate (EGCG) with a content of 75 - 95%; after the refining 13,7 g of EGCG was obtained: white pink needle-shaped crystal, ESI-MS spectrum m/z = 458.7 corresponding to the molecular weight = 458; $C_{22}H_{18}O_{11}$; Melting point: 216^{o} C.



***epi*-Gallocatechin gallate**
(2*R*,3*R*)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl 3,4,5-trihydroxybenzoate

Chemical shifts of epi-gallocatechin gallate

No	**δ_H (ppm)**	**δ_C (ppm)**
2	4,95 *brs*	76,54 *d*
3	5,37 *brs*	68,06 *d*
4	Ha: 2,66 *d* *J* = 17,5 Hz Hb: 2,93 *dd* *J* = 5 Hz; 17,5 Hz	25,78 *t*
5	9,27 *brs* (Ar-O<u>H</u>, C5)	156,52 *s*
6	5,93 *d* *J* = 2 Hz	95,59 *d*
7	9,03 *brs* (Ar-O<u>H</u>, C7)	156,57 *s*
8	5,83 *d* *J* = 2,5 Hz	94,39 *d*

9	- - -	155,66 *s*
10	- - -	97,45 *s*
1’	- - -	128,69 *s*
2’ & 6’	6,41 *brs* (2 H)	105,56 *d* (2 C)
3’ & 5’	8,68 *brs* (2 Ar-O<u>H</u>, C3’ & C5’)	145,67 *s* (2 C)
4’	7,98 *brs* (Ar-O<u>H</u>, C4’)	132,41 *s*
-COO-	- - -	165,26 *s*
1’’	- - -	119,37 *s*
2’’ & 6’’	6,81 *brs* (2 H)	108,74 *d* (2 C)
3’’ & 5’’	9,16 *brs* (2 Ar-O<u>H</u>, C3’’ & C5’’)	145,43 *s* (2 C)
4’’	8,86 *brs* (Ar-O<u>H</u>, C4’’)	138,59 *s*

Solvent DMSO-d6

For the first time, the high-pressure liquid chromatography using Diaion HP20 chromatographic separation system connected online with Sephadex LH20 chromatography gel column was applied and tested in the industrial production in Vietnam. The new technology has shown the good separation efficiency of EGCG and catechins in the green tea, obtained products have a high purity> 95%. The method has the advantage in term of the short chromatographic separation time with the high separation efficiency, time saving, the commont solvents such as ethanol/water, not harmful in the food industry have been used. The method is perfectly suitable for use in the industrial production.

3.3. Semi - synthesis of o-acetyl derivative of catechins in the green tea and the assessement of the degree of degradation of the free radical scavenging activity

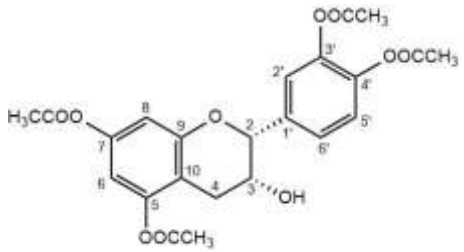
3.3.1. Semi - synthesis of the acetyl derivatives of catechin in the green tea to assess the level of the activity decline

The activity of clearing the free radicals of catechins in the green tea is higher than that of antioxidants such as vitamin C, vitamin E, resveratrol .... But these catechins are easy to degrade by the temperature and light, which limits their applicability a lot.

Investigation of the semi synthesis of the epi-catechin O-acetyl derivatives (EC), epi-gallocatechin (EGC) and epi-gallocatechin gallate (EGCG) using acetic anhydride with the simple, easy reaction conditions. Based on this, the possible degradation of these derivatives has been evaluated.

3.3.1.1. Substance epi-Catechin acetate (I)

Substance I crystallized in the ethyl acetate, having a white needle-shaped crystals, melting point 142-144° C, ESI-MS m/z = 458.5 corresponding to molecular weight = 458; chemical formular: $C_{23}H_{22}O_{10}$. Results of the nuclear magnetic resonance spectroscopy (1D) and 2D NMR showed that the structure is perfectly compatible with (2R, 3R) -5,7-O-diacetyl-3 ', 4'-O-diaceticlepicatechin (EC acetate).



Chemical shifts (δ) of epi-Catechin acetate

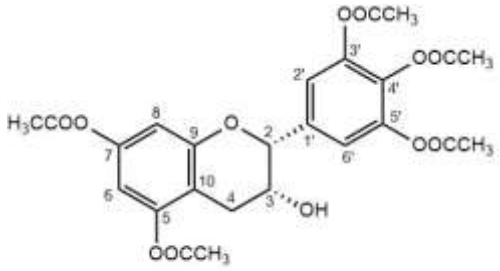
No	δ_H (ppm)	δ_C (ppm)
2	5.17 *d* *J* = 5 Hz	77,93 *d*
3	4,19 *m*	63,35 *d*
4	Ha: 2,6 *m* Hb: 2.8 *m*	28,81 *t*
5	- - -	149,98 *s*
6	6,62 *d* *J* = 2,5 Hz	107,43 *d*
7	- - -	149,07 *s*

8	6,57 *d J* = 2,5 Hz	108,43 *d*
9	- - -	111,46 *s*
10	- - -	79,19 *s*
1’	- - -	137,91 *s*
2’	7,41 *d J* = 2 Hz	122,16 *d*
3’	- - -	141,59 *s*
4’	- - -	141,36 *s*
5’	7,28 *m*	123,05 *d*
6’	7,37 *d J* = 2 Hz	125,16 *d*
C=O (C5)	- - -	155,24 *s*
C=O (C7)	- - -	168,36 *s*
C=O (C3’)	- - -	168,54 *s*
C=O (C4’)	- - -	169,05 *s*
CH_3 (COO-C5)	2,24 *br* (3H)	20,34 *q*
CH_3 (COO-C7)	2,28 *br* (3H)	20,38 *q*
CH_3 (COO-C3’)	2,29 *br* (3H)	20,49 *q*
CH_3 (COO-C4’)	2,30 *br* (3H)	20,49 *q*

Solvent $CDCl_3$

3.3.1.2. Substance epi-gallocatechin acetate (II)

Substance II crystallized in the ethyl acetate having a white needle-shaped crystals, melting point 114-170° C; ESI-MS m / z = 516.1 corresponding to molecular weight = 516; chemical formula: $C_{25}H_{24}O_{12}$. The result of the nuclear resonance spectral analysis for the structure is perfectly consistent with (2R, 3R) -5,7-O-diacetyl-3 ', 4', 5'-O-triacetylepigallocatechin (EGC acetate).



***epi*-Gallocatechin acetate**
(2*R*,3*R*)-5,7-O-diacetyl-3',4',5'-O-triacetylepigallocatechin

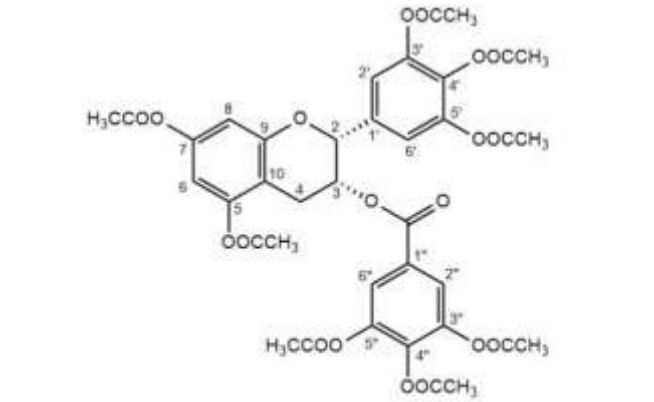
Chemical shifts (δ) of epi-Gallocatechin acetate

No	δ_H (ppm)	δ_C (ppm)
2	5.01*s*	77,79 *d*
3	4,26 *m*	65,15 *d*
4	2,83 *m* 2,90 *m*	28,36 *t*
5	- - -	154,76 *s*
6	6,66 *d J* = 2,5 Hz	107,96 *d*
7	- - -	150,06 *d*
8	6,56 *d J* = 2,5 Hz	108,84 *d*
9	- - -	149,67 *s*
10	- - -	110,10 *s*
1’	- - -	134,38 *s*
2’ & 6’	7,25 *br* (2 H)	119,01 *d* (2 C)
3’ & 5’	- - -	136,41 *s* (2 C)
4’	- - -	143,51 *s*
C=O (C5)	- - -	166,92 *s*
C=O (C7)	- - -	168,46 *s*
C=O (C3’ & C5’)	- - -	167,92 *s* (2C)
C=O (C4’)	- - -	168,91 *s*
CH_3 (COO-C5)	2,27 *s* (3H)	20,16 *q*
CH_3 (COO-C7)	2,29 *br* (3H)	20,61 *q*
CH_3 (COO-C3’&C5’)	2,29 *br* (6H)	20,74 *q* (2C)
CH_3 (COO-C4’)	2,296 *br* (3H)	21,07 *q*

Solvent $CDCl_3$

3.3.1.3. Substances epi-gallocatechin gallate, O-octaacetyl (III)

Substance III crystallized in the ethanol, having a white needle-shaped crystal, melting point 102-105 ° C, ESI-MS m/z = 795 [M + H] correspond to molecular weight = 794, chemical formular C38H34O19. The results of the nuclear magnetic resonance spectra analysis for structure are perfectly consistent with (2R, 3R) -5,7-di-O-acetyl-3 ', 4', 5'-triacetyl-epigallocatechin-3-O - (3 ', 4' ', 5' '- O-triacetyl) -gallate (EGCG acetate).



***epi*-Gallocatechin gallate, O-octaacetyl**
(2*R*,3*R*)-5,7-di-O-acetyl-3',4',5'-triacetylepigallocatechin-3-O-(3",4",5"-O-triacetyl)gallate

Chemocal shifts of EGCG acetate

No	δ_H (ppm)	δ_C (ppm)
2	5,06 *br*	76.84 *d*
3	5.43 *br*	67,59 *d*
4	Ha: 2,76 *d* *J* = 17,5 Hz	25,99 *t*
	Hb: 3,00 *dd* *J* = 3,5 Hz; 23,5 Hz	
5	- - -	156,39 *s*
6	6,20 *m*	100,82 *d*
7	- - -	155,39 *s*
8	6,20 *m*	101,06 *d*
9	- - -	149,52 *s*
10	- - -	104,27 *s*
1’	- - -	128,22 *s*
2’ & 6’	6,42 *m* (2 H)	105,55 *d* (2 C)
3’ & 5’	- - -	145,44 *s* (2 C)
4’	- - -	132,51 *s*
-COO-	- - -	165,19 *s*
1’’	- - -	119,16 *s*
2’’ & 6’’	6,83 *m* (2 H)	108,72 *d* (2 C)
3’’ & 5’’	- - -	145,43 *s* (2 C)
4’’	- - -	138,65 *s*
C=O (C5)	- - -	168,57 *s*
C=O (C7)	- - -	169,05 *s*

C=O (C3’ & C5’)	- - -	169,05 *s* (2 C)
C=O (C4’)	- - -	172,05 *s*
C=O (C3’’ & C5’’)	- - -	165,19 *s* (2 C)
C=O (C4’’)	- - -	172,05 *s*
$\underline{CH_3}$COO (C4’ & C4’’)	1,91 *br* *	20,7 *q* *
$\underline{CH_3}$COO (Ar)	2,23 - 2,27 *m* *	20,5 *q* *

Solvent DMSO-d_6

** Signal imposed*

3.3.2. Evaluation of the degradation level of the radical scavenging activity of O-acetyl derivatives compared to catechins in the green tea.

The antioxidant activity has been carried out by DPPH radical scavenging ability in the Department of Applied Biochemistry, Institute of Chemistr under Vietnamese Academy of Science and Technology.

Results of survey of DPPH free radical scavenging activity of Catechins and O-acetyl derivatives

No	**Radical sample**	**EC_{50} radical Catechin**	**EC_{50} of Catechin acetate**
1	epi-Catechin	7,08 µg/ml	45,64 µg/ml
2	epi-Gallocatechin	4,60 µg/ml	52,99 µg/ml
3	epi-Gallocatechin gallate	5,00 µg/ml	34,58 µg/ml
4	Positive control from resveratrol	8,23 µg/ml	8,23 µg/ml

EC_{50}: Half maximal effective concentration

The results of the antioxidant activity showed that epi-catechin acetate had an activity 7.5 times less then the original EC, epi-gallocatechin acetate has an active 10 times less than EGC; EGCG acetate degrades the activity at least ~ 7 times compared to

EGCG but is still the most active. The antioxidant activity of these derivatives although decreased but remained high enough with an EC50 value of 34.58 - 52.99 μg/ml. The water solubility of this derivative is almost unchanged: epi-catechin acetate is still water-insoluble such as EC, derivatives of EGC acetate and EGCG acetate are well soluble in water.

3.4. Investigation of the process to obtain caffeine from the green tea

Caffeine crystalline, white; ESI-MS m/z = 195 [M + H] +; correspond to molecular weight = 194; chemical formular $C_8H_{10}N_4O_2$; melting point: 238^o C. Analysis by SKLM has a Rf ~ 0.9L photoluminescence under ultraviolet light of 360 nm (TLC - Solvent system: Chloroform/methanol/citric acid 0.5% by volume ratio of 3: 2: 0,2).

Chemical shifts of caffeine

No	δ_H (ppm)	δ_C (ppm)
2	7,97 *s* (1H)	142,73 *d*
4	---	106,56 *s*
5	---	154,50 *s*
7	---	151,01 *s*
9	---	148,07 *s*
10	3,86 *brs* (3H)	33,05 *q*
12	3,19 *brs* (3H)	27,40 *q*
14	3,39 *brs* (3H)	29,30 *q*

Chemical structure of caffeine

CONCLUSION

The thesis has investigated the extracting/separating technology of the major catechins of Vietnamese green tea (Camellia sinensis L.) in order to contribute to the develop of the pharmaceutical and food industry. The thesis has obtained the following results:

1. A technology process on the pillot level to extract catechins in the green tea by using a continuous liquid/solid back flow extraction technique has been set up. The procees when compared with the ethanol extraction method by Soxhlet method gave the result: The concentration of EGCG in catechins was 50.62% when extracted with ethanol and was 53.67% when extracted by water; But the yield of total catechins extracted by water reached 12.5% higher than extracted by ethanol (9,4%). The continuous back flow extraction by water has the highly advantage in term of scale, time, solvent costs and biological safety.

This is the first time in Vietnam a pillot-scale process using the continuous liquid/ solid back flow extraction has been carried out and set up in the aim to seperate catechins from the green tea.

2. The separation efficiency of chromatographic methods for the separation of EGCG with various chromatographic phases such as the normal, the reverse phase, the adsorption/desorption have been investigated and compared. Based on the ratio of total catechins/column diameters multiplied the seperation efficiency to select the specific column for EC, EGC, ECG, EGCG from the total catechin. A purification process of catechins from the total catechins of the green tea using HPLC high performance liquid chromatography was performed using Diaion HP20 SS and Sephadex LH 20 with a separation efficiency of more than 90%, catechins have a purity of ≥ 95%, quality suitable for the pharmaceutical uses.

For the first time, the high pressure liquid chromatography using Diaion HP20 chromatography separator and chromatography column Sephadex LH20 was applied and tested in the semi-industrial production in Vietnam.

3. The ability of the free radical scavenging activity EC_{50} of catechins in the green

tea has been evaluated with EC, EGC, EGCG respectively 7,08; 4,60; 5,00 μg/ml. The result showed that hydrogen-aren groups in the catechin structure play an important role in the antioxidant activity of these substances.

4. The semi-synthesis of O-acetyl derivatives of EC, EGC and EGCG catechins has been investigated without the use of pyridine as solvent. The free radical scavenging of acetate derivatives has been evaluated and compared and compared to the original catechins, resveratrol. The acetyl derivatives have a reduced the free radical scavenging activity against radical catechins but remain high: EC O-acetyl; ECG O-acetyl and EGCG O-acetyl have an EC50 value of 45.64 μg / ml respectively; 52.99 μg / ml and 34.58 μg/ml. The decrease of the free radical scavenging activity of the O-acetyl catechins increases the stability of these substances.

5. The modern methods of HPLC/MS; 1D and 2D NMR have been applied to analyze and to determine the structure and purity of catechins and O-acetyl derivatives of EC, EGC and EGCG. The result showed these compounds have the correspond structure, reaching a purity of ≥ 95%.

LIST OF WORKS PUBLISHED BY THE AUTHOR

1. ***Do Thanh Ha***, Mai Thanh Nga, Hoang Van Hoan, Nguyen Quoc Dat, Ngo Thi Hai Yen, Pham Thi Thanh Hieu (2012), "*Investigation on the production technology of epi-gallocatechin gallat (EGCG) 95%*", Journal of Chemistry, T. 50 (6), tr 727-731;

2. Đoan Thi Van, Byxteeva U.M, Bataeva D.C., ***Do Thanh Ha*** (2012), "*The antimicrobial properties of substances extracted from the green tea on Yersinia enterocolitica microorganism*", Journal of the Problems of Modern Biology, Matxcova, T. 3, tr. 82-84;

3. ***Do Thanh Ha***, Pham Thi Thanh Hieu, Hoang Van Hoan, Tran Thi Nhu Mai, Giang Thi Phuong Ly (2017), "*The semi-synthetic studies and assessments of the depleting level of the free radical scavenging activity of some derivatives of acyl catechins in the green tea*", Journal of Chemistry, in progress.