

PERPUSTAKAAN UMP



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DETERMINATION OF S DE A IN STEVIA
REBAUDIANA LEAVES VIA PREPARATIVE HPLC

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ABSTRACT

Stevia rebaudiana leaves, also known as sweet leaf. It has been used as sweetener for centuries in South America. *Stevia rebaudiana* leaves contain non-caloric sweeteners (steviol glycosides) whose consumption could exert beneficial effects on human health. Steviol glycosides are considered safe. The aim of present study was to determine qualitative of stevioside and rebaudioside A which compounds of steviol glycosides consist in *Stevia* leaves by soxhlet extraction method using Waters preparative HPLC autopurification system. Samples were sequentially extracted by soxhlet extraction method using methanol solvent and the analytes separated by Waters XBridge C₁₈ column (150 mm x 4.6 mm I.D., 5µm). This HPLC analytical column, performed with a mobile phase in isocratic mode elution consisting of acetonitrile/water (80:20 v/v). Stevioside were the most abundant steviol glycosides found followed rebaudioside A in samples of *Stevia* (n = 5) from Malaysia. This proposed Waters preparative HPLC autopurification system can be applied for further analysis in this study for the routine quality control of *Stevia* leaves and their commercial preparations.

ABSTRAK

Daun Stevia rebaudiana, juga dikenali sebagai daun manis. Ia telah digunakan sebagai bahan pemanis berabad lamanya di Amerika Selatan. Daun Stevia rebaudiana mengandungi, pemanis tanpa kalori (steviol glycosides) yang pengambilannya boleh memberikan kesan yang menguntungkan pada kesihatan manusia. steviol glycosides dianggap selamat. Objektif kajian ini, untuk menentukan kuantitatif dan kualitatif stevioside and rebaudioside A yang terdapat pada sebatian steviol glycosides di mana ianya terkandung dalam daun Stevia dan ianya dihasilkan dengan menggunakan kaedah pengeskrakan soxhlet serta di analisis oleh Waters preparative HPLC sistem autopurification. Penghasilan sampel berturut –turut dengan methanol yang dilakukan dengan menggunakan kaedah pengeskrakan soxhlet dan analytes dipisahkan oleh Waters XBridge C₁₈ kolum (150 mm x 4.6 mm I.D., 5µm). HPLC analytical kolum ini, akan dijalankan dengan mobile phase dalam keadaan isocratic yang mengandungi acetonitrile/sir (80:20 v/v). Stevioside merupakan pemanis yang paling banyak dalam sebatian steviol glycosides, diikuti rebaudioside A ke atas sampel daun Stevia (n = 5) dari Malaysia. Dicadangkan Waters preparative HPLC autopurification system boleh digunakan untuk melanjutkan kajian ini bagi pengendalian rutin kawalan kualiti ke atas daun Stevia dan secara komersialnya.

TABLE OF CONTENTS

	Page
SUPERVISOR'S DECLARATION	ii
STUDENT'S DECLARATION	iii
ACKNOWLEDMENT	v
ABSTRACT	vi
ABSTRAK	vii
TABLE OF CONTENTS	viii
LIST OF TABLES	xii
LIST OF FIGURES	xiii
LIST OF SYMBOLS	xv
LIST OF ABBREVIATIONS	xvi
CHAPTER 1 INTRODUCTION	
1.1 Background of Study	1
1.2 Problem Statements	6
1.3 Objectives	8
1.4 Scope of Study	8
CHAPTER 2 LITERATURE REVIEW	
2.1 Background of Stevia Rebaudiana Leaves	10
2.2 About Steviol Glycosides	12
2.3 Soxhlet Extraction Method	17
2.4 Preparative HPLC Instrument Method for Analysis	20

TABLE OF CONTENTS

	Page
SUPERVISOR'S DECLARATION	ii
STUDENT'S DECLARATION	iii
ACKNOWLEDMENT	v
ABSTRACT	vi
ABSTRAK	vii
TABLE OF CONTENTS	viii
LIST OF TABLES	xii
LIST OF FIGURES	xiii
LIST OF SYMBOLS	xv
LIST OF ABBREVIATIONS	xvi
CHAPTER 1 INTRODUCTION	
1.1 Background of Study	1
1.2 Problem Statements	6
1.3 Objectives	8
1.4 Scope of Study	8
CHAPTER 2 LITERATURE REVIEW	
2.1 Background of Stevia Rebaudiana Leaves	10
2.2 About Steviol Glycosides	12
2.3 Soxhlet Extraction Method	17
2.4 Preparative HPLC Instrument Method for Analysis	20

CHAPTER 3 METHODOLOGY

3.1	Experimental Method	24
3.2	Material and Chemical Reagent	25
3.3	Equipment	25
	3.3.1 Soxhlet Extractor	25
	3.3.2 Preparative HPLC	27
3.4	Sample Preparation	28
3.5	Analysis Method Preparative HPLC	29
	3.5.1 Mobile Phase Preparation	30
	3.5.2 Standard Solution Preparation	31
	3.5.3 Sample Preparation	31

CHAPTER 4 RESULT AND DISCUSSION

4.1	Sample Preparation	32
	4.1.1 Stevioside and Rebaudioside A	32
	4.1.2 Soxhlet Extraction Method of Stevia Leaves Sample Solutions	34
4.2	Optimization of Preparative HPLC Conditions for Analysis	34
4.3	Preparative HPLC Column	36
4.4	Qualitative Determination of Stevioside and Rebaudioside A	37

CHAPTER 5 CONCLUSIONS AND RECOMMENDATIONS

5.1	Conclusions	42
5.2	Recommendations	43

REFERENCES	46
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APPENDICES		48
A1	Chromatogram Result HPLC – Methanol	48
A2	Data Result HPLC – Methanol	49
B1	Chromatogram Result HPLC – Stevia Sample (m = 1.0g)	50
B2	Data Result HPLC – Stevia Sample (m = 1.0g)	51
C1	Chromatogram Result HPLC – Stevia Sample (m = 2.5g)	52
C2	Data Result HPLC – Stevia Sample (m = 2.5g)	53
C3	Data Result HPLC – Stevia Sample (m = 2.5g)	54
D1	Chromatogram Result HPLC – Stevia Sample (m = 3.0g)	55
D2	Data Result HPLC – Stevia Sample (m = 3.0g)	56
D3	Data Result HPLC – Stevia Sample (m = 3.0g)	57
E1	Chromatogram Result HPLC – Stevia Sample (m = 5.0g)	58
E2	Data Result HPLC – Stevia Sample (m = 5.0g)	59
E3	Data Result HPLC – Stevia Sample (m = 5.0g)	60
F1	Chromatogram Result HPLC – Stevia Sample (m = 10.0g)	61
F2	Data Result HPLC – Stevia Sample (m = 10.0g)	62
F3	Data Result HPLC – Stevia Sample (m = 10.0g)	63

G1	Chromatogram Result HPLC – Rebaudioside A and Stevioside Standard Solution	64
H1	Chromatogram Result HPLC – Stevia Sample and Rebaudioside A Standard Solution	65
H2	Chromatogram Result HPLC – Stevia Sample and Stevioside Standard Solution	66
I1	Chromatogram Result HPLC – Stevioside Standard Solution Concentration	67
I2	Chromatogram Result HPLC – Rebaudioside A Standard Solution Concentration	68

LIST OF TABLES

Table No		Page
3.1	List of Samples Data	28
4.1	List of Five Samples Result Data for Area Percentage	33
4.2	Condition Operating Waters Preparative HPLC Autopurification System	36

LIST OF FIGURES

Figure No.		Page
1.1	Dried Stevia rebaudiana leaves	2
1.2	Chemical structure of steviol glycosides	2
1.3	Soxhlet extractor apparatus	3
1.4	Preparative HPLC Waters Autopurification™ System instrument	4
1.5	Waters HPLC Column XBridge C ₁₈ (150mm x 4.6mm I.D., 5µm)	5
1.6	Flowchart research of determining the SV and Rb A compounds in Stevia rebaudiana leaves using preparative HPLC	9
2.1	Stevia rebaudiana leaves	10
2.2	Chemical structure of steviol glycosides	12
2.3	Chemical structure of stevioside	14
2.4	Chemical structure of rebaudioside A	14
2.5	Structures nine known of related compounds to the steviol glycosides	15
2.6	Dried Stevia rebaudiana leaves	16
2.7	Soxhlet extraction method	18
2.8	Preparative HPLC Waters Autopurification™ System instrument method	20
2.9	Chromatogram of mixture nine steviol glycosides standard solution using Capcell pak C ₁₈ MG II Column	23

3.1	Illustration of soxhlet extractor	26
3.2	Diagram preparative HPLC instrument	27
3.3	Flowchart of sample preparation	29
3.4	Flowchart of preparative HPLC analyses	30
3.5	Filtering mobile phase using mobile phase filtration equipment	31
4.1	Graph different mass samples against area percentages	33
4.2	Chromatogram of stevioside standard solution for 1 ppm concentration	37
4.3	Chromatogram of rebaudioside A standard solution for 1 ppm concentration	38
4.4	Chromatogram of stevioside and rebaudioside A in Stevia sample	38
4.5	Chromatogram of mixture nine steviol glycosides standard solution using Capcell pak C ₁₈ MG II Column	41

LIST OF SYMBOLS

NH ₂	Amino
C ₁₈	Carbon-18
°C	Degree Celcius
m	Mass
n	Number
pH	Negative logarithm of the hydrogen ion concentration
P	Pressure

LIST OF ABBREVIATIONS

ATR	Attenuated Total Reflectance
CE	Capillary Electrophoresis
DuA	Dulcoside A
ELS	Evaporative Light Scattering
FDA	Food and Drug Administration
FIR	Fourier Infrared
FT-IR	Fourier Transform Infrared
GRAS	Generally Recognized as Safe
HPLC	High Performance Liquid Chromatography
ID	Internal Diameter
JECFA	Joint European Commission on Food Additives
LC	Liquid Chromatography
M.wt	Molecular weight
MCE	Membrane Cellulose Ester
MS	Mass Spectroscopy
NIR	Near Infrared
NMR	Nuclear Magnetic Resonance
RbA	Rebaudioside A
RbB	Rebaudioside B
RbC	Rebaudioside C
RbF	Rebaudioside F
Rf	Response factor
RuB	Rubusoside
RP	Reverse Phase
Stb	Steviolbioside
SIR	Selected Ion Recording

SPE	Solid Phase Extraction
ST	Steviol
SV	Stevioside
UK	United Kingdom
US	United State
UV	Ultraviolet
ZnSe	Zinc Selenide

CHAPTER 1

INTRODUCTION

1.1 Background of study

Stevia rebaudiana leaves as an herbaceous perennial plant native of Paraguay and Brazil. This wondrous herb is also known as “Sweet Weed”, “Honey-Leaf”, “Sweet-Leaf” and “Sweet-Herb” and which is estimated to be 300 times sweeter than can sugar (Chalapathi, M.V. et al., 1997). However, *Stevia rebaudiana* leaf is the only species to have the natural sweetness to replace artificial sweeteners.

Stevia leaves sweeteners are available commercially in many forms including dried leaves (**Figure 1.1**), purified powders and liquid extracts. *Stevia rebaudiana* leaves extracts are used medicinal plants besides as low-calorie sweetener in Japan since 1968, and subsequently have been introduced in other countries such as Brazil, Indonesia, Korea, Mexico, Tanzania, Singapore, Thailand, China, USA and since 1990 in Canada.



Figure 1.1: Dried *Stevia rebaudiana* leaves

Stevia rebaudiana leaves which are extracts can be sold as a dietary supplement. The extracts have been used for sweetening soft drinks such as diet coke, soy sauce, dried seafood, candies, ice-cream, chewing gum, yoghurt, and as well as in toothpaste and mouthwash in Japan, Korea, and Brazil (Erkucuk, A. et al., 2009).

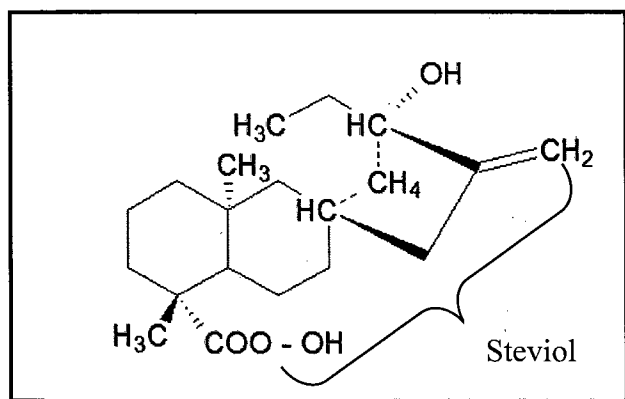


Figure 1.2: Chemical structure of steviol-glycosides (SG)

Stevia rebaudiana plant is one of 154 members of the genus *Stevia* and one of two species that produce sweet steviol glycosides (**Figure 1.2**) (Madan, S. et al., 2010). It consist about nine active components of steviol glycosides (SG) which are they, stevioside (SV), rebaudioside A (RbA), rebaudioside B (RbB), rebaudioside D (RbD), rebaudioside F (RbF), steviolbioside (Stb), rubusoside (Rub), rebaudioside C (RbC) and dulcoside A (DuA). Each of them contributes their own percentage of sweet flavor to the *Stevia* leaf.

While, according to the research of determining the two active compounds (SV and RbA) of Stevia leaves, one of the best ways is by extracting among the several types of extraction method such as soxhlet extraction, hydro distillation, ultrasonic, and others.

The soxhlet extraction (**Figure 1.3**) is the most conventional of all methods and consists of a simple distillation process repeated a number of times. Soxhlet extraction method is a straight forward method. The sample phase is always in contact with fresh solvent, thereby enhancing the displacement of target compound from the matrix and the compound are not decomposed due to the moderate extraction condition.

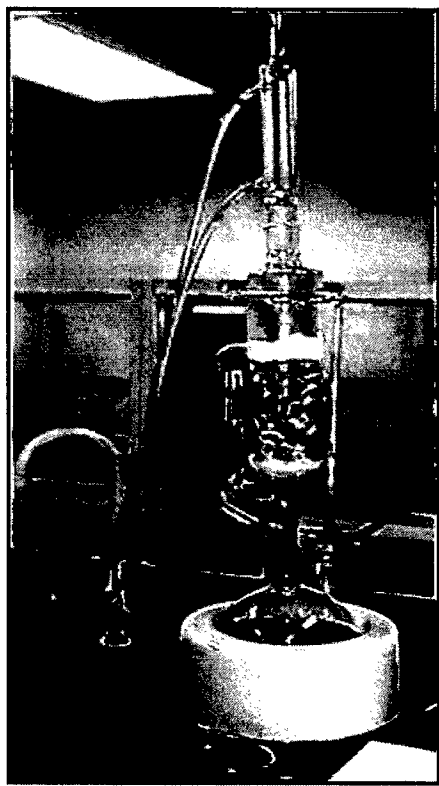


Figure 1.3: Soxhlet extractor apparatus

In this study, the experimental procedure for this research in *Stevia rebaudiana* leaves focus on one main instrument method which is preparative High Performance Liquid Chromatography (HPLC) for study the qualitative analysis of the SV and RbA compounds content in *Stevia* leaves.

HPLC instrument is a solution phase technique for fractionation of complex samples. Once introduced to the column, it is the sample's differential affinity for the solvent (mobile) and stationary phases that enables separation to occur, generally with sharper resolution than standard liquid chromatography.

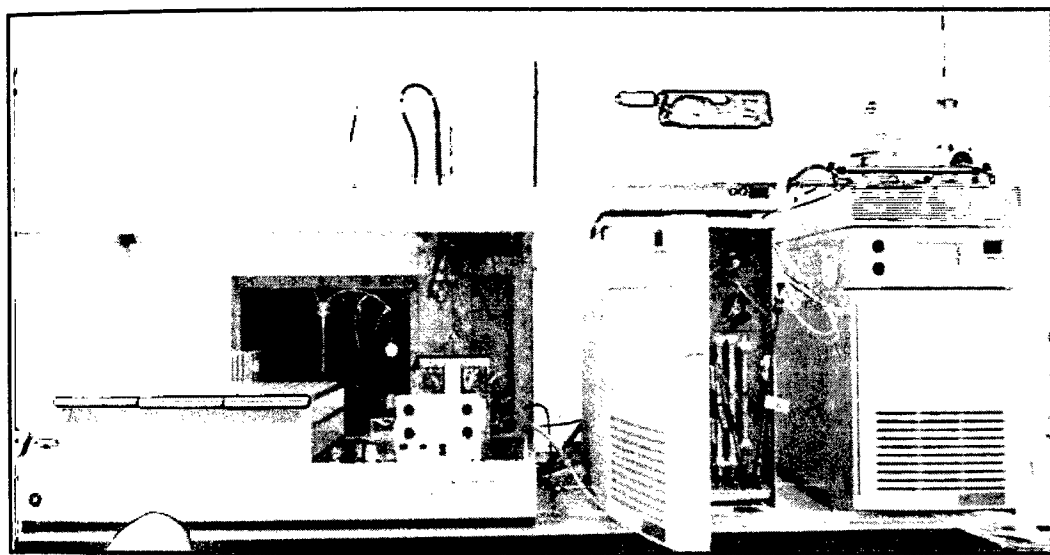


Figure 1.4: Preparative HPLC Waters AutoPurification™ System Instrument

HPLC can be used for analytical or preparative applications depending on the HPLC pump and the column size. HPLC columns are available in a variety of formats, including reverse phase, ion exchange, and size exclusion, which separate based on polarity, charge, and size, respectively.

The preparative HPLC Waters AutoPurification™ System with the UV/Vis detector (**Figure 1.4**) is used in this research. It is well known as versatile purification and good isolation solution among other Waters HPLC models.

This HPLC have a lot of benefits, but the major of among its advantages, have a combination of integrated high-performance instrumentation.

In addition, it gives robust and reliable system which is improved the ability to manage ever increasing workload demands. It also flexible configurations that enables easy scale-up from analytical to preparative chromatography. This Waters® Purification Systems reliably isolate and purify microgram to multigram quantities of the sample compounds of interest. Besides, it also can run a few samples a day or several hundred, which it will operate with level of confidence and productivity that multi-vendor systems cannot match.

While for the most important component role play in the HPLC, is the HPLC column itself. So for this research, using the analytical column named Waters XBridge C₁₈ column, with features; 4.6 mm ID x 150 mm, 5µm (**Figure 1.5**). This column has own unique itself which is it gives maximizing column efficiency. This XBridge packing incorporate the use of well-characterized, state of the art, proprietary procedures for bonding and endcapping, that make it show very little retention loss and exhibit a column lifetime equivalent to that sterically hindered C₁₈ silica bonded phase.

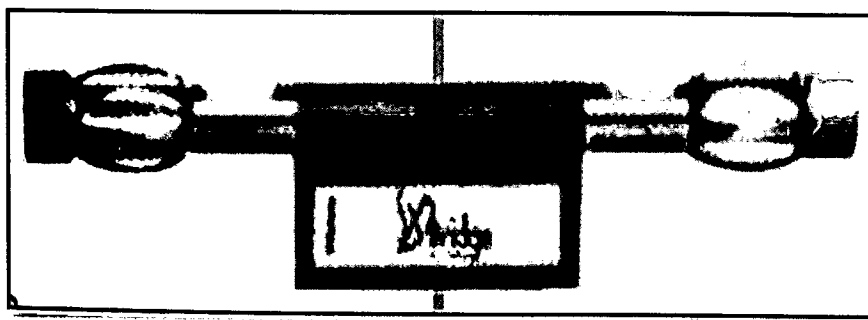


Figure 1.5: Waters HPLC Column XBridge C₁₈,
(150 mm x 4.6 mm ID, 5 µm)

This special XBridge column is designed under accelerated pH 10 stability test conditions, a direct comparison to some of the most popular chromatographic phases, that claimed to have extended high pH stability, which it clearly shows this column lifetime exceeding that of best silica column by over 1000% with very little degradation in chromatographic performance.

This column has also been shown to the maximum stability in the widest range of volatile and non volatile buffer types. In addition, it also designed to eliminate this compromise and deliver the flexibility to work under any mobile phase, temperature and pH conditions, thus make it speeding up the process to an optimum and rugged final method.

About the mobile phase selection for this study, the bonded stationary phase is nonpolar in nature and is best used with the mobile phase such as methanol/water or acetonitrile/water mixtures, but in this situation the mobile phase of acetonitrile/water mixtures was used.

The reason is increasing the amount of organic component, usually reduces the retention time of the sample. Gradient elution techniques for this packing of use 5% methanol or acetonitrile in water as the initial solvent, and 100% methanol or acetonitrile as the final solvent as depending to the solvent use as the mobile phase on the research.

1.2 Problems statements

Currently, *Stevia rebaudiana* leaves have very high demand and prices in the world market for its popular uses in medicine and as a sweeter of drinks. However, there is limited research on *Stevia* leaves extraction in the world. *Stevia* leaf is also used in production of pharmaceutical products widely.

Steviol glycosides extraction industry has a big potential to grow up in Malaysia. At this moment, there is no steviol glycosides extraction industry in Malaysia. In the research of report on "China's Stevia Extract Industry, (2011-2012)", state that, now Malaysia becomes the major export market for China's Stevia rebaudiana extracts with over half of the export shares. However, Malaysia is not the ultimate consumer market of Stevia rebaudiana extracts, but a transit base.

Even though Stevia leaves are quite expensive, the extraction of steviol glycosides can yield. Besides that, the soxhlet extraction also not really safe for human health because it uses organic solvent large quantity such as methanol and hexane. It can cause hepatitis and kidneys malfunction. Soxhlet extraction also required higher cost of extraction because the uses of large amount organic solvent.

The demand for increasingly clean and efficient chemical synthesis is continuously becoming more urgent from both an economic and an environmental standpoint. So-called green technologies are looking for alternatives yet they focus on large quantities of hazardous even toxic solvents. One could ever say that the best solvent is no solvent.

Conventional method of extractions that is soxhlet extraction that applies heat principle is rare methods that are no people used, today. The methods will yield many impurities in the solution extracted, needs long period of extraction and yield the low strength of present the nine active compounds of steviol glycosides.

However, a new green technique to extract steviol glycosides known as pre-treatment is Solid Phase Extraction (SPE) was commonly used in recent years that make the extraction simpler and give best extraction of steviol glycosides. The technique will yield better quality of steviol glycosides production, higher percentage yield and reduce the number of non-impurities during the extraction. Besides, this technique prevents solvent wastes, hazards and toxicity.

1.3 Objectives

There are two main objectives of this study.

- I. To determine the stevioside and rebaudioside A compounds contain in Stevia rebaudiana leaves using preparative HPLC instrument method.
- II. To determine the qualitative analysis based on the retention time of the stevioside and rebaudioside A compounds by soxhlet extraction method using preparative HPLC.

1.4 Scope of study

The scopes of this study are essentially to investigate the preparative HPLC instrument method. There are some important tasks to be carried out in order to achieve the objectives of this study. The important scopes have been identified for this research in achieving the objectives:

- I. In this research, the samples of Stevia rebaudiana leaves will be used to extract. This extraction will undergo soxhlet extraction method.
- II. In this study, qualitative analysis of the extraction Stevia leaf will be done to determine the SV and RbA compounds present in this Stevia leaf. The present of both active compounds will be identified via preparative HPLC. By doing so, to determine the both active compounds in Stevia leaf will be identified.

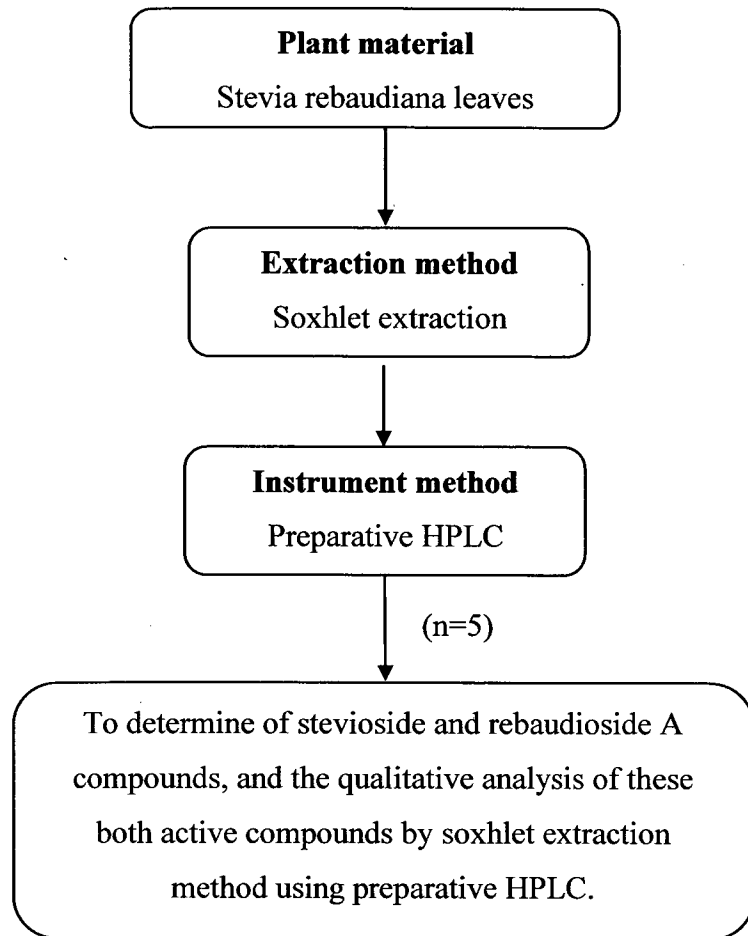


Figure 1.6: The flowchart research of determining the SV and RbA compounds in *Stevia rebaudiana* leaves using preparative HPLC.

CHAPTER 2

LITERATURE REVIEW

2.1 Background of Stevia Rebaudiana Leaves



Figure 2.1: Stevia rebaudiana leaves

A zero calorie plant known for its sweetness is Stevia rebaudiana leaves (**Figure 2.1**). It is a member of plant in the Chrysanthemum family. It grows wild as a tiny shrub in Brazil and Paraguay a long time ago.