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Stability of Steviosides in Beverages and Emulsions

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Contents

Abstract

Consuming large amount of sugars, especially sucrose, has resulted in the increased prevalence of diseases, such as diabetes, obesity, cardiovascular diseases, and dental problems (Kobus-Moryson & Gramza-Michalowska, 2015).Therefore, in the last couple of decades, artificial sweeteners are used in large quantities in food industry as sugar substitutes. The artificial sweeteners belong to the class of high-intensity sweetener (HIS). They are many times sweeter then sucrose and have reduced calorie content (Kim & Kinghorn, 2002). The sweeteners such as aspartame, neotame, acesulfame-K, saccharin and sucralose contain very low amount of calories, and are produced synthetically (Anton et al., 2010).

Therefore, scientists are developing natural sweeteners as alternative for sucrose, but with similar properties (Gonzalez et al., 2014).

In the last couple of years, stevia, ″the sweet herb from Paraguay″, attracts attention of many researchers (Soejarto, 2002; Chatsudthipong & Muanprasat, 2009; Goyal & Goyal, 2010; Mishra et al., 2010; Thomas & Glade, 2010; Yadav et al., 2011).

Stevia represents a good alternative to sucrose, because it is natural, and 300 times sweeter than sucrose (Soejarto et al., 1983), and a non-caloric sweetener.

Steviol glycosides which are found in stevia leaves, are responsible for the sweetness of stevia (Genus, 2000).

Today, extracts of stevia leaves, steviol glycosides, are used in some Asian countries and in South America as a natural, low-calorie sweetener, in some products such as soft drinks, yoghurt, soja and others.

Some researcher have found that stevia has beneficial effects on human health, including prevention of diabetes (Suanarunsawat & Chaiyabutr, 1997; Toskulkao et al., 1995), antihypertensive (Chan et al., 2000; Lee et al., 2001), antihyperglycemic (Jeppesen et al., 2000, 2002) and, non-cariogenic (Das et al., 1992) properties.

Emulsions are systems which containa at least two liquids that do not mix perfectly (Becher, 1965; Gopal, 1969; Tadros et al., 1983). Emulsions are widely used in food, pharmaceutical and cosmetics industry (Schwarz et al., 2000).

They are unstable systems, which can be stabilized by adding emulsifier. An emulsifier is a substance which is responsible for emulsion stability and formation of droplet aggregates. To find a good emulsifier which will enable emulsion stability for a long time is a field of active research.

In this study, the stability of rebaudioside A, rebaudioside B, rebaudioside D and rubusoside during incubation at different temperatures, pH values and storage times were analyzed. The second task was to observe steviosides behavior in emulsions (milk, oil and espresso).

Incubation of the steviosides in citric acid buffer at room temperature and refrigerator temperature showed good stability in pH range 3-6 up to one week of incubation. After one week of incubation decreasing steviosides stability was noticed. Stevioside incubation in phosphoric acid buffer at room temperature and refrigerator temperature showed good stability in pH range 3-5 up to one month incubation. Steviosides are remarkably stable in a pH range 3-5 under thermal treatment up to 95 °C and 50 minutes of incubation.

In the experiment about steviosides behavior in emulsions, results showed that steviosides tends to stay in the aqueous phase. In milk and espresso only rebaudioside D could be detected. It is of importance to study more about steviosides behavior in emulsions.

1. Introduction

The artificial sweeteners are used in large amount in food industry as additive (De et al., 2013). *˝The global market for high - potency sweeteners during 2010 was reported to be $ 1. 146 billion˝* (Leatherhead Food Research, 2011). The artificial sweeteners are sugar substitutes, mostly synthetically produces. They are popular because they are sweeter than sugar and they have reduced calorie content (Mitchell, 2006; Nabors, 2011; Wilson, 2007). Today, large popularity gained naturally sweeteners, stevioside, derived from plant *Stevia rebaudiana*. In the figure 1.1 are shown stevia production in the word, in the last couple of years.

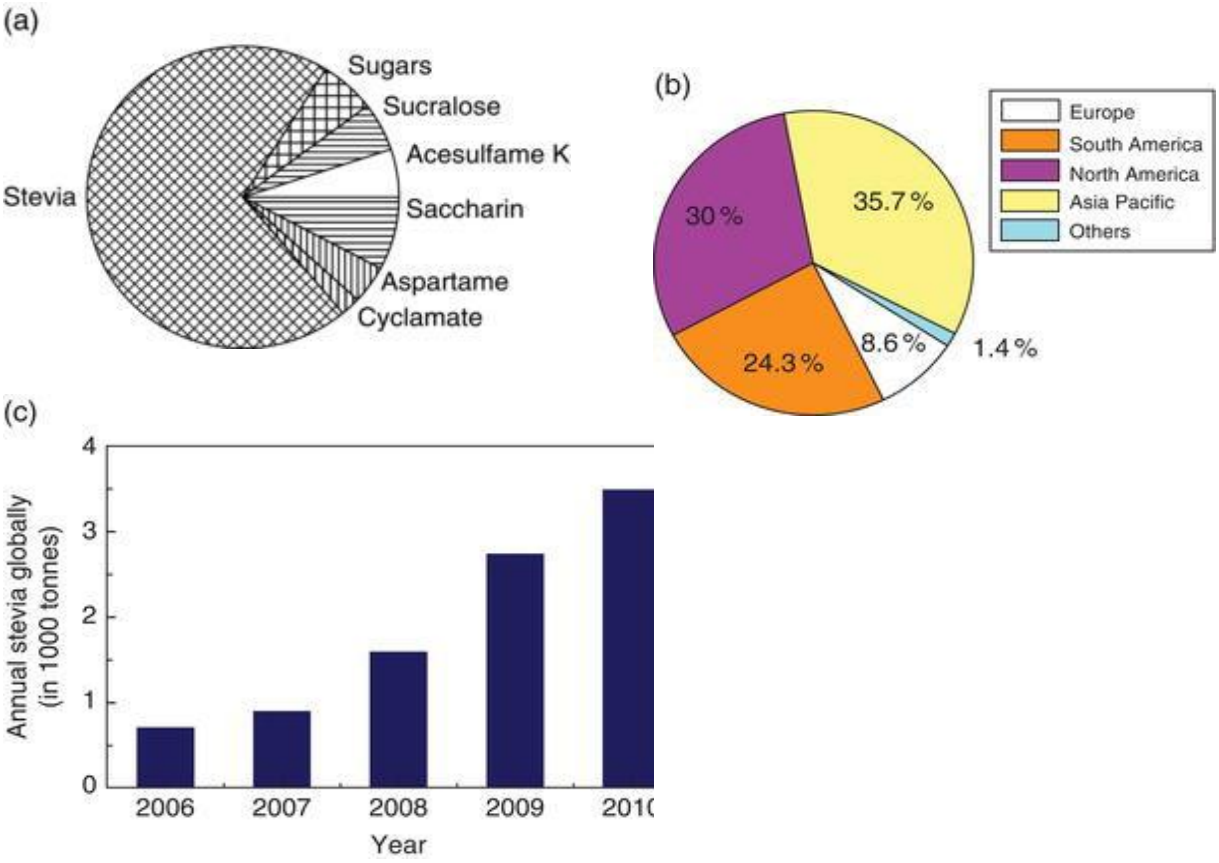


Figure 1.1 Stevia productions in the world *(*from De et al., 2013).

Stevia rebaudiana Bertoni belong to the *Asteraceae* family. It grows in South America (Katayama et al., 1976).

Traditionally, the plant was used as medicines and to sweeten the drinks. In the 1960s Japan began growing the stevia as a crop (Sumida, 1968). Stevia extracts were first used in Japan in 1970s, for sweetening the different types of food and beverages.

Figure 1.2 *Stevia rebaudiania* Bertoni plant

http://www.motherearthnews.com/organic-gardening/herbs/stevia-plant-zm0z13fmzkin.aspx

One of the main importance of stevia is that, its leaves produce glycosides (stevioside and rebaudioside), which are up to 300 times sweeter then sucrose. Another benefit is that they are 100 % natural and have no calorie; it is used as an alternative for sucrose (Soejarto et al., 1982 and 1983).

Some of the artificial sweeteners are found in different food and beverages (Puri et al., 2011). Demand for natural and healthier options, with reduced calorie content, high sweetness and solubility, put stevia plant in the middle of attention in the last couple of years.

1.1 History of *Stevia rebaudiana*

The homeland of stevia is Latin America-Paraguay (Soejarto, 2002; Yadav et al., 2011; Lemus-Mondaca et al., 2012), but it could be growth in other areas, in South-America (Soejarto, 2002), and in Asia and Europe (Amzad-Hossain et al., 2010; Gardana et al., 2003).

In the 16s century the Guarani Indians had used plant to sweet the food and for medicines, they knew advantage of this plant, which they called ‘kaa he-he’, which means ‘sweet herb’, (Soejarto et al., 1983; Jeppesen et al., 2002; 2003).

Moisés Santiago Bertoni, scientifically described stevia in 1889. At first stevia was called *Eupatorium rebaudianum*, and afterwards in 1905 it is changed to *S. rebaudiana* Bertoni, in honor of Santiago Bertoni (Barriocanal et al., 2008).

Stevioside was isolated in 1909 and extract was first time purified in 1931. Few decades later several other compounds were isolated, including the most abundant, rebaudioside A, which is sweeter then stevioside (Barriocanal et al., 2008). Structurally, stevioside molecule contains steviol core (aglycon) and three glucose molecule attached to the steviol.

Stevia leaves produce sweet ent-kaurene diterpenoid glycosides, which give the stevia it sweet taste. There are more than 200 species identified but only two species *rebaudiana* and *phlebophylla* produce steviol glycosides (Brandle & Telmer, 2007). Stevia contains 4-20 % steviol glycosides of the dry weight (Ghanta et al., 2007).

The habitat of *Stevia rebaudiana* is subtropical and tropical area in the Southern Hemisphere, with temperatures about 23 °C (Brandle & Rosa, 1992).

1.2 Description of stevia plant

Fig 1.3 Stevia rebaudiana Bertoni

http://sweetgreenfields.com/our-story/about-stevia/

Stevia rebaudiana is a small ancient bush from *Asteraceae* family. It does not rise high, and it could be growth worldwide (Mishra et al., 2010). Stevia possess small leaves, approximately 3-4 cm long, (Shock, 1982), with straight steam and branching roots (Lemus-Mondaca et al., 2012). It yields white small flowers with 2-6 florets (Blumenthal, 1996; Katayama et al., 1976). Stevia tolerates different kind of soils, sandy, damp, dark.

Ultimately, for the cultivation *Stevia rebaudiana* prefers soils, with enough percent of moist, but not too much (Mishra et al., 2010; Lemus-Mondaca et al., 2012). In 1943 the stevia was first cultivated in UK, then in 1968 in Japan (Lewis, 1992), today productions are extend to countries all over the world, such Mexico, Brazil, India, Canada and Korea (Brandle & Rosa, 1992; Fors, 1995; Yadav et al., 2011). Stevia grows in almost every part of the world, particularly in South America (Sivaram & Mukundam, 2003).

Stevia rebaudiana is cultivated as annual herb, in sunny regions. The stevioside content in leaves depends on the length of the day (Lemus-Mondaca et al., 2012).
Stevia rebaudiana is perennial plant, and for growth needs temperature between 20-24 °C, it does not tolerate the cold (Singh & Rao, 2005). Consequently, for the cultivation stevia needs lot of water because in the case of prolonged stress stevia wilt rapidly, which is one of the limitations for the cultivation (Lemus-Mondaca et al., 2012).

1.3 Therapeutic effects of stevia

Stevia rebaudiana could be the crop of the future, especially because of high sweetness and supposed therapeutics effects (anti-tumor, anti-hyperglycemic and diuretic). Japan first used stevioside as a sweetener in food industry. After that, use of the stevia as a low-calorie sweetener expend to other Asian countries, such China, Malaysia, Singapore, Taiwan and Thailand (Chatsudthipong & Muanprasat, 2009).

Extracts of the stevia (steviol glycosides) are used as alternative for sucrose, for prevention diabetes mellitus, obesity and caries (Pól et al., 2007).

Stevia leaves has superior properties in comparison to other artificial sweeteners, it natural, non–nutritive, 300 times sweeter than sugar, could be applied in prevention of *˝modern˝* diseases (Goyal et al., 2010).

Savita, Sheela, Sunanda, Shankar, and Ramakrishna (2004) found that stevia leaves supply the body with only 2.7 kcal/ g (dry weight). It is justified the number one of low-calorie sweetener, in comparison to other available sweeteners such as saccharin, aspartame and sucralose (Savita et al., 2004).
Some of the potential health benefits of stevioside are outlined in Table 1.1.

Table 1.1 Different physiological effects of stevioside consumption
(from De et al., 2013)

Health effects	References
Antihyperglycemic effect (reduction in blood glucose level both type 1 and type 2 diabetes)	Jeppesen et al., 2000,2002,2003
Anticarcinogenic effect	Mizushina et al., 2005
Natural antioxidant	Shukla et al., 2009; Ghanta et al., 2007
Enhances glucose metabolism	Suanarunsawat & Chiayabutr, 1997;Toskulkao et al., 1995
Antidiarrheal therapeutics	Tomita et al., 1997; Pariwat et al., 2008
Ulceration in the gastrointestinal tract	Kochikyan et al., 2006
Decreases the sugar content,cholesterol, triglyceride	Atteh et al., 2008
Anti- inflammatory effect	Jayaraman et al., 2008; Sehar et al., 2008
Antimicrobial effect	Puri & Sharma 2011;Takahashi et al., 2001
Diuretic	Melis1995

1.4 Steviol glycosides extraction

In the last decades, the extraction processes of the steviol glycosides attract attention of many scientists. To get the highly quality extract from stevia leaves, extraction process consist of multiple operations (De et al., 2013). This operation include: pretreatment, separation, purification and refining (Munish et al., 2011).

One of the most often used operations is extraction with hot water, but it shows some of the drawbacks including: long time and high temperatures (Dacome et al., 2005). Additionally, extraction can be performed with ion exchange (Unesh et al., 1977), solvent extraction (Haga et al., 1976), adsorption chromatography (Itagaki & Ito, 1979) and solvent plus a decolorizing agent (Ogawa et al., 1980). Because of the complicity this extractions processes, the influence on the stevioside yield's from extract is not known (Munish et al., 2011). Currently, an extraction with enzymes becomes an interesting topic, because it offers a many advantages compared to other techniques. For the enzyme-extraction is needed less time and energy and in the end high yield extract is obtained (Yang et al., 2010).

1.5 Steviol glycosides (SG's)

Steviol glycosides are found in the leaves of *Stevia rebaudiana* plant. They belong to the group of ent-kaurene diterpene glycosides (Lukasz Woznaik et al., 2014). Guarani Indians used this plant for centuries, but it took long time to be discovered and used worldwide (Misra et al., 2011). Steviol glycosides give stevia it sweet taste, which are about 300 time sweeter then sucrose.

˝Structurally, stevioside (13 – [2 – O – β – D – glucopyranosyl – α – glucopyranosyl) - oxy] kaur – 16-en -19 – oic – acid – β - D - glucopyranosyl ester) is a glycoside with a glucosyl and a sophorosyl residue attached to the aglycone steviol. ˝ (Munish et al., 2011).

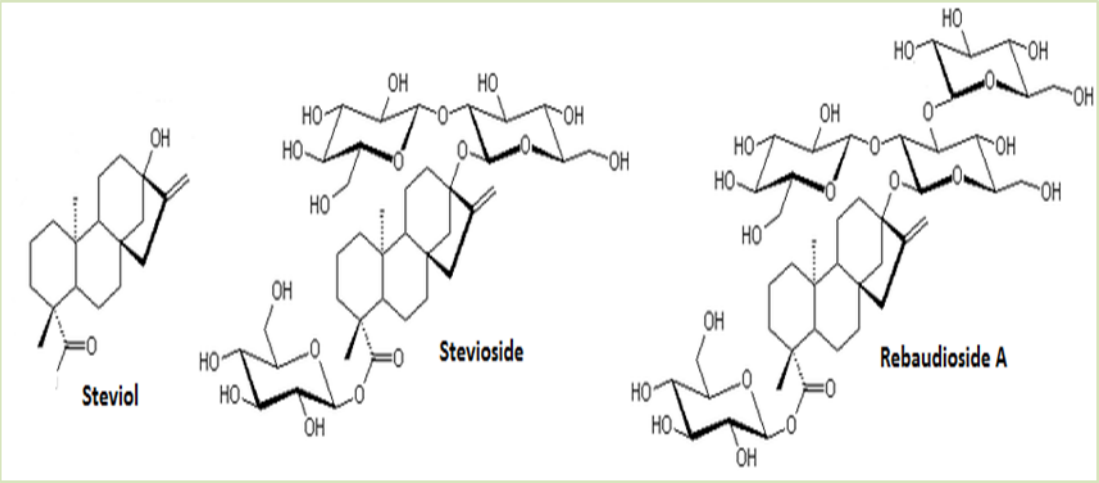
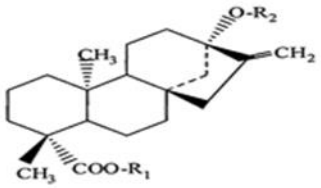


Figure 1.4 Structure of steviol, rebaudioside A and stevioside
https://examine.com/supplements/stevia/

It has be found that *Stevia rebaudiania* leaves contain eight different steviol glycosides, this are: steviolbioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F and dulcoside A. All steviosides have a common steviol backbone, the only difference between different steviol glycosides is the number and type of sugar molecules attached to the steviol core, at position C13 and C19 (Kenelly, 2002; Kolb et al., 2001). The structures of steviol glycosides are illustrated in Figure 1.5.



Compound name	R_1 (C-19)	R_2 (C-13)
1. Steviol	H	H
2. Steviolmonoside	H	β-Glc
3. Rubusoside	β-Glc	β-Glc
4. Steviolbioside	H	β-Glc-β-Glc(2→1)
5. Stevioside	β-Glc	β-Glc-β-Glc(2→1)
6. Rebaudioside A	β-Glc	β-Glc-β-Glc(2→1) β-Glc(3→1)
7. Rebaudioside B	H	β-Glc-β-Glc(2→1) β-Glc(3→1)
8. Rebaudioside C (Dulcoside B)	β-Glc	β-Glc-α-Rha(2→1) β-Glc(3→1)
9. Rebaudioside D	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) β-Glc(3→1)
10. Rebaudioside E	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)
11. Rebaudioside F	β-Glc	β-Glc-β-Xyl(2→1) β-Glc(3→1)
12. Dulcoside A	β-Glc	β-Glc-α-Rha(2→1)

Figure 1.5 Structure of the major glycosides of *Stevia rebaudiana* leaves. Glc, Xyl, and Rha represent, respectively, glucose, xylose and rhamnose sugar moieties (from Genus, 2003).

Two compounds are found in the highest concentration in the stevia leaves, namely rebaudioside A and stevioside, which make together about 90 % total glycosides mass (Ceunen et al., 2013). Rebaudioside A has implied physical properties, it is the sweetest and most stable in comparison to other glycosides. Stevioside have bitter taste, hereby the crude extract from leaves is bitter too (Dacome et al., 2005). Extracts with higher concentration of rebaudioside A have better tasting properties. Additionally, the sweetness of other glycosides is lower (Camer & Ikan, 1987).

Some of the benefits of the stevioside is that it is not synthetic, having reduced calories content, do not cause dental problems, stay stable at high temperatures and is a flavor enhancer (Goyal et al., 2010). Besides, use of steviol glycosides as sweeter is found to be safe for human health. Another important advantage is that it can be used in prevention of diabetes mellitus, because of reduced calorie content (Geuns et al., 2006; Huxtable, 2002).

Besides the glycosides stevia contains other compounds in different concentrations, such as flavonoids, oils, (De et al., 2013), proteins, (Abou-Arab et al., 2010; Mohammad et al., 2007), water soluble vitamins (Kim et al., 2011) and fatty acid (Tadhani & Subhash 2006). Table 1.2 shows content of stevia leaves.

Table 1.2 Proximate analysis of dry *S. rebaudiana* leaves
(from De et al., 2013)

Component	% (w/w) of dry weight basis
Moisture	4.2-6.5
Protein	6.2-20.4
Fat	2.5-5.6
Crude fibre	13.6-18.5
Ash	8.5-13.1
Carbohydrates	35.2-52.8
Reducing sugar	5.6-6.1
Non-reducing sugar	9.6-9.9

The cultivation conditions influence the stevioside content in dried leaves of *Stevia rebaudiania*. Approximately 5 % to 20 % are found in the dried leaves (Kim & Dubois 1991). Steviol glycosides are found in flowers, approximately 0.9 – 1 % (w/w) (Darise et al., 1983). Additionally, they can be found in stalks, seeds and roots (Kobus-Moryson et al., 2015). Stevioside is found in stevia stalks at lower concentrations (Yadav et al., 2011).

In table 1.3 are listed comparison of steviol glycosides from stevia leaves. The most abundant, stevioside (5-10 %) and rebaudioside A (2-4 %) are found in highest concentration, then follows rebaudioside C with (1-2 %), and dulcoside A (0.2-0.7 %) (Genus, 2003).

Table 1.3 Comparison of sweet glycosides present in *S. rebaudiana* (from De et al., 2013)

Diterpene Glycoside	Content (%)	Relative sweetening power	Molecular mass (g/mol)	Reference
Stevioside	5.0-10.0	250-300	804.87	Bridel & Lavielle, 1931a
Rebaudioside A	2.0-4.0	350-450	967.01	Sakamoto et al., 1977
Rebaudioside B	<< 1.0	300-350	804.87	
Rebaudioside C	1.0-2.0	50-120	951.01	
Rebaudioside D	<<1.0	200-300	1129.15	
Rebaudioside E	<<1.0	250-300	967.01	
Rebaudioside F	<<1.0	nd	936.99	
Steviolbioside	<<1.0	100-120	642.73	Kohda et al., 1976
Dulcoside A	0.4-0.7	50-120	788.87	Kobayashi et al., 1977

nd - not defined

Stability of steviol glycosides have key role in food industry, besides all other advantages. It has been found that stevioside is very stable molecule, which tolerates high temperatures and undergoes degradation at temperatures above 140 °C. For whole molecule to be degraded the temperature at 200 °C is needed. Thereby it can be used in processed food (Kroyer, 2010).

Table 1.4 details the physical properties for all the glycosides. They are poorly soluble in water. But rebaudioside A which has been found in higher concentration in stevia leaves, and its sweeter then stevioside, showed good solubility in water because rebaudioside A contains four sugar molecules attached to the steviol core (Kinghorn & Soejarto 1991; Kohda et al., 1976)

Table 1.4 Physical properties of steviol glycosides present in *S. rebaudiana* (from De et al., 2013)

Compound	Melting point (°C)	Solubility in water (%)
Stevioside	196-198	0.13
Rebaudioside A	242-244	0.80
Rebaudioside B	193-195	0.10
Rebaudioside C	215-217	0.21
Rebaudioside D	283-286	1.00
Rebaudioside E	205-207	1.70
Steviolbioside	188-192	0.03
Dulcoside A	193-195	0.58

As steviol glycosides are not hydrolyzed by mouth bacteria, they reach the colon intact. Afterwards, steviol glycosides are metabolized by gut bacteria, to the aglycon (steviol) and sugar molecules. Steviol is then absorbed into the blood stream and transported to the liver. Furthermore, steviosides are conjugated in the liver to steviol glucuronide. At the end, metabolized steviosides are excreted by urine (Gardana et al., 2003).

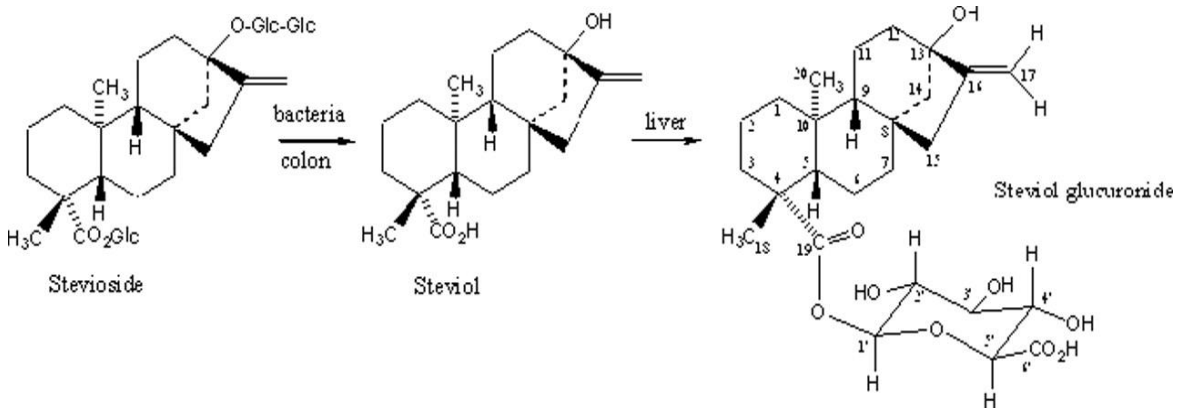


Figure 1.6 Hypothetical routes from dietary stevioside to steviol glucuronide in human urine (Genus et al., 2007)

1.6 Applications in food and beverages

In the 21st century the world's population is faced with health problems, such as obesity, diabetes and cardiovascular diseases. A sharp rise in these diseases is a consequence of consuming high-calorie food and beverages, and lack of physical activity (Anton et al., 2010; Puri et al., 2012). Therefore, the increased attention is being focused on sweeteners with reduced sugar content (Brahmachari et al., 2011; Pečivová et al., 2013; Reguła & Kowalewska, 2010).

Additionally, in the last decades products with inscription ″natural″, ″zero calorie″, ″without added sugars″ achieved increased demand. Currently, *Stevia rebaudiania*, with being natural, 300 times sweeter then sucrose, low calories content is an ideal replacement for sugar and others artificial sweeteners (Anton et al., 2010; Puri et al., 2012).

Extracts from stevia leaves, steviol glycosides have a wide application in different foods such as: desserts, beverages, confectioneries, yoghurt, fruit products and dietary supplements. They are ideal for processed products, because they are more heat stable, compared to some others sugar substitute (Mehrotra et al., 2014).

Japan used stevia as a sweetener in many products in the last 40 years (Chatsudthipong & Muanprasat 2009; Thomas & Glade, 2010).

The European Union approved in December 2011, steviol glycoside as food additive with E number - 960, for use in 31 food categories (Commission Regulation, 2011).

Table 1.5 shows use of steviol glycosides in different foods.

Table 1.5 Food use levels of steviol glycosides reported to the sixty-third meeting of JECFA (Joint (FAO/ WHO) Exert Committee on Food Additives).
(from Mehrotra et al., 2014)

Food type	Maximum use level reported (mg/kg)
Beverages	500
Desserts	500
Yogurt	500
Cold confectionery	500
Sauces	1000
Pickles	1000
Delicacies	1000
Sweet corn	200
Bread	160
Biscuits	300

1.7 Emulsions

Emulsions are systems which contain at least two liquids that do not mix perfectly (Becher, 1965; Gopal, 1969; Tadros et al., 1983). Emulsions are widely used in food, pharmaceutical and cosmetics industry (Schwarz et al., 2000).

Generally, each emulsion contain two phase, dispersed and continuous phase. There are different types of emulsions, the simples is: water-in-oil (W/O) or oil-in -water (O/W) emulsion. In the water-in-oil emulsions the water droplets are dispersed in oil, and in oil-in-water emulsions the oil droplets are dispersed in water phase (Becher, 1965; Gopal, 1969; Tadros et al., 1983; Lin et al., 1975; Froster et al., 1997; Sudol et al., 1997; Nakajima et al., 1997). Different emulsions are prepared due to stirring of two liquids, and their properties are influenced by composition, temperature, pressure, and used method such as which component is added first (Lin et al., 1975; Froster, 1997; Esquena et al., 1998).

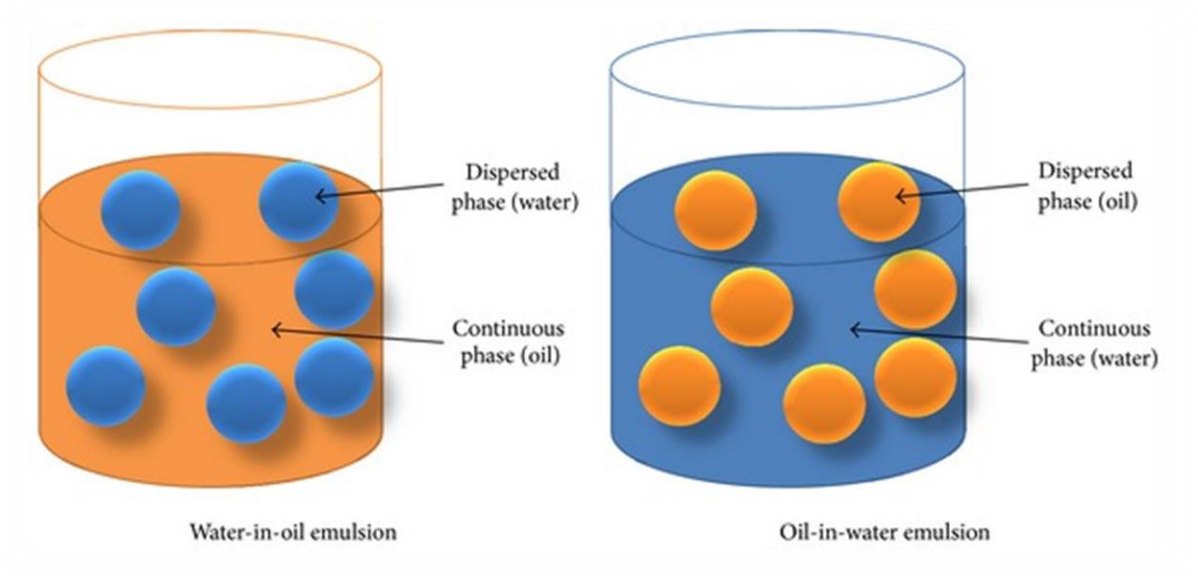


Figure 1.7 Concept of two-phase water-in-oil and oil-in-water emulsions

https://www.researchgate.net/figure/260376328_fig2_Concept-of-two-phase-water-in-oil-and-oil-in-water-emulsions

Emulsions are unstable systems which tend to break down during storage by variety processes such as coalescence, flocculation, creaming and breaking.

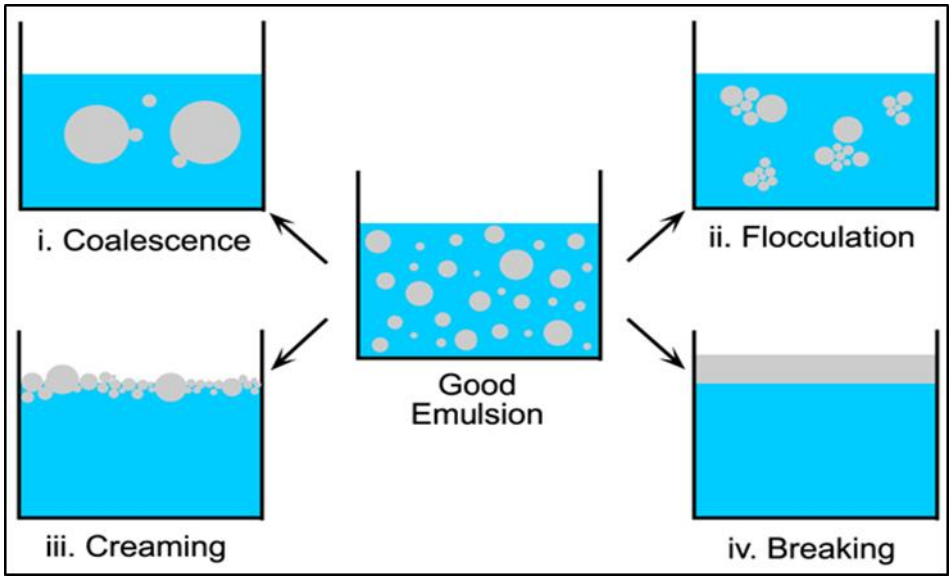


Figure 1.8 The break down processes of emulsions
http://cubecinema.com/cola/chemistry/cola1.htm

I. **Coalescence -** is the process in which more liquid droplets sticks together and formed a larger one
II. **Flocculation -** is the process in which droplets merged together and formed aggregates
III. **Creaming -** is the process in which droplets make a foam on the top of the emulsions
IV. **Breaking -** is the process in which two layer are formed, water and oil

In the production of emulsions an emulsifier is used (Friberg & Larsson, 1997; McClements, 1999; Stauffer, 1999).
An emulsifier is a substance that enables two immiscible liquids to form a homogenized mixture. It is adsorbed on the droplet surface (Stauffer, 1999; Walstra, 1996; Charlambous, 1989; Krog et al., 1997), and it decreases droplet size due to the lowering the interfacial tension between water and oil (Walstra, 1993).

Emulsions stability and droplet aggregation depend on emulsifier, which forms a thin layer around the droplet (Friberg & Larsson, 1997; McClements, 1999). Some emulsifiers have ability to make small droplets but their disadvantage is that they formed emulsions which are unstable at long-term storage (Stang et al., 1994).

Qualities of a good emulsifier are: rapid adsorption on the droplet surface, reducing the surface tension and prevention droplets to aggregate for a long time (McClements, 1999; Walstra, 1993; Stang et al., 1994).

Emulsifiers can be divided into two groups: synthetic and natural. They are phospholipids, proteins, polysaccharides (Stauffer, 1999; Charlambous, 1989; Krog, 1997).

Beverage emulsions are water-in-oil emulsions. Components that make oil phase are flavor oils, weighting agents,vegetable oil and antioxidant, whilst the water phase is consist of emulsifier, sweetener, salts and acids (Trubiano, 1995; McClements, 1999). The most common physical insability of beverages emulsions are known as ″ringing″ and ″oiling-off″ (Trubiano, 1995; Tan, 1997, 1998), which are results of sedimentation, flocculation, and coalescence (Dickinson & Stainsby, 1982; Dickinson, 1992; McClements, 1999).

2. Material and Methods

During my work, two experiments were conducted. The first investigated steviosides stability at different pH values, different temperatures and different storage times. The second one was to observe the distribution of steviosides in emulsions.

2.1. Materials

Highly purified steviol glycoside, rebaudioside A, rebaudioside B, rebaudioside D and rubusoside were obtained from Red Bull GmbH (Austria). All other samples milk, oil and espresso were provided from Spar (Austria). In a first experiment for testing, the steviosides stability, the steviosides were dissolved in ACN:H_2O (1:1) at a concentration of 1 mg/ml. For studying the steviosides distribution in emulsions, the concentrations used were 0.5 and 2 mg/ml.
Table 2.1 lists all chemicals used in my studies.

Table 2.1 Chemicals and reagents

Chemicals	Producer
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \times H_2O$)	Merck GmbH
Acetonitrile (CH_3CN)	Chem-Lab NV
Phosphoric acid (H_3PO_4)	Chemist Plomer
Citric acid water free ($C_6H_8O_7$)	Merck GmbH
Disodium phosphate dihydrate ($Na_2HPO_4 \times 2H_20$)	Merck GmbH
Citric acid monohydrate ($C_8H_8O_7 \times H_20$)	Roth GmbH
Sodium hydroxide (NaOH 0.1 M)	Carl Roth GmbH + Co

2.2 Methods of analyses

The steviosides were determined by HPLC. For the analysis two APS-2 HYPERSIL columns were connected in series (150 × 4.6 mm + 50 × 4.6 mm). The Thermo Scientific™ Hypersil™ APS-2 Amino LC columns analyze sugars.
The separation of the 10 µl samples was performed within 15 min at a flow rate 1 ml/min and a column temperature of 25 °C. The samples were separated isocratically using (a mixture of 75 % ACN with 25 % $NaH_2PO_4 \times H_2O$ pH 3), detection at 210 nm wavelength.

2.3 Sample preparation

For the stability analysis the steviosides were used at concentrations of 1 mg/ml as mentioned before. At first two types of buffer with different pH values were prepared:

- **Citric acid buffer**
- **Phosphoric acid buffer**

2.3.1 Preparation of citric acid buffer

Citric acid buffer was prepared, by McIlvaine, as follows

Table 2.3.1.1 Approach of the stock solutions

A) Citric acid water free	19.21 g	with distilled H_2O fill-up to 1000 ml
B) Disodium phosphate dihydrate	35.60 g	with distilled H_2O fill-up to 1000 ml
A) Citric acid monohydrate	21.01 g	with distilled H_2O fill-up to 1000 ml

http://www.aeisner.de/rezepte/puffer1.html

Table 2.3.1.2 Buffer solutions

pH	Disodium phosphate dihydrate	A) Citric acid water free	A) Citric acid monohydrate
2.6	10.8 ml	+ A fill up to 100 ml	
3	20.6 ml	+ A fill up to 100 ml	+ A fill up to 100 ml
4	38.6 ml	+ A fill up to 100 ml	+ A fill up to 100 ml
5	51.5 ml	+ A fill up to 100 ml	+ A fill up to 100 ml
6	63.2 ml	+ A fill up to 100 ml	
7	82.4 ml	+ A fill up to 100 ml	

http://www.aeisner.de/rezepte/puffer1.html

2.3.2 Preparation of phosphoric acid buffer

For the preparation of phosphoric acid buffer 75 % phosphoric acid was used. The phosphoric acid was diluted 1:20 with water. And then the desired pH=3, pH=4.2 and pH=5 was adjusted with sodium hydroxide 0.1 M.

2.3.3 Stability of steviosides at room and refrigerator temperature

1 mg rebaudioside A, rebaudioside B, and rebaudioside D were dissolved in 1 ml ACN:H_2O (1:1), and 1 ml rubusoside was mixed with 1 ml ACN:H_2O (1:1), and then incubated in citric acid and phosphoric acid buffer different pH values at 23 °C and 6 °C up to one week in citric acid buffer and up to one month in phosphoric acid buffer. Samples were taken after one, three, seven and thirty days, and then analyzed by HPLC.

2.3.4 Stability of steviosides at elevated temperatures

Rebaudioside A, rebaudioside B, rebaudioside D and rubusoside were dissolved in ACN:H_2O (1:1) at a concentration 1 mg/ml. Than 200 µl solution was added to 200 µl citric acid buffer and 200 µl phosphoric acid buffer different pH values and incubated in glass vials at different temperatures from 60 °C up to 95 °C for 50 minutes. After incubation the samples were cooled on ice and then 100 µl ACN was added. Afterwards the samples were analyzed by HPLC. Rubusoside was mixed with citric acid buffer pH 2.6, 3, 4, 5, 6 and with phosphoric acid buffer pH 3, 4.2 and 5, in proportion 1:1. Afterwards the solutions were incubated at 72 °C up to 10 min, then cooled on ice, 100 µl ACN added, and analyzed by HPLC.

2.3.5 Steviosides behavior in emulsions

For the analysis of steviosides distribution in emulsions 0.5 mg of steviosides (rebaudioside A, rebaudioside B and rebaudioside D) were dissolved in 1 ml milk (fat content 3.5 %). Then centrifuged 20 minutes at 14 000 rpm at 4 °C. After that, 300 µl of the aqueous phase were taken and mixed with 700 µl ACN, and once more centrifuged for 20 minutes. Afterwards, 500 µl were taken for HPLC analysis.

Emulsion of rape seed oil and water were stirred for 10 minutes, and then 700 µl was taken and mixed with 700 µl of steviosides solutions, concentrations of 0.5 mg/ml. This solution was then centrifuged for 20 minutes at 14 000 rpm at 4 °C, and analyzed by HPLC.

And the last experiment was done in espresso coffee. Steviosides solutions in concentration 2 mg/ml were mixed with espresso in ratio 1:1. Afterwards, the same procedure as with milk was performed.

3. Results

Table 3.1 Limit of detection (LOD) and limit of quantification (LOQ) for rebaudioside A, B and D. Concentration reb. A and B 1.25 g/L, reb. D 2.5 g/L,

Signal	LOQ [g/L]	LOD [g/L]
Reb. A	2.1	0.6
Reb. B	1.4	0.4
Reb. D	2	0.6

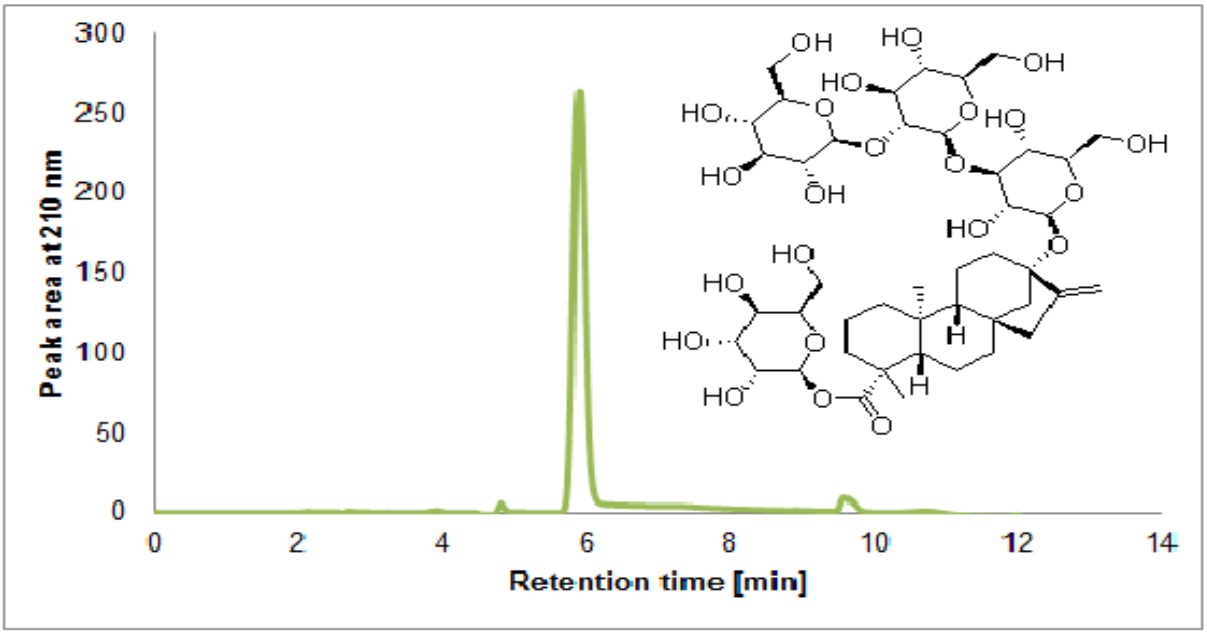


Figure 3.1 Chromatogram of rebaudioside A, under separation (75 % ACN, 25 %10 mM $NaH_2PO_4 \times H_2O$, pH=3) at a flow rate of 1 ml/min. Injection volume was 20 µl, pressure 115 bar, temperature 25 °C and stop time 12 min. Column APS-2 HYPERSIL (150 × 4.6 mm + 50 × 4.6 mm); concentration 1 mg/ml

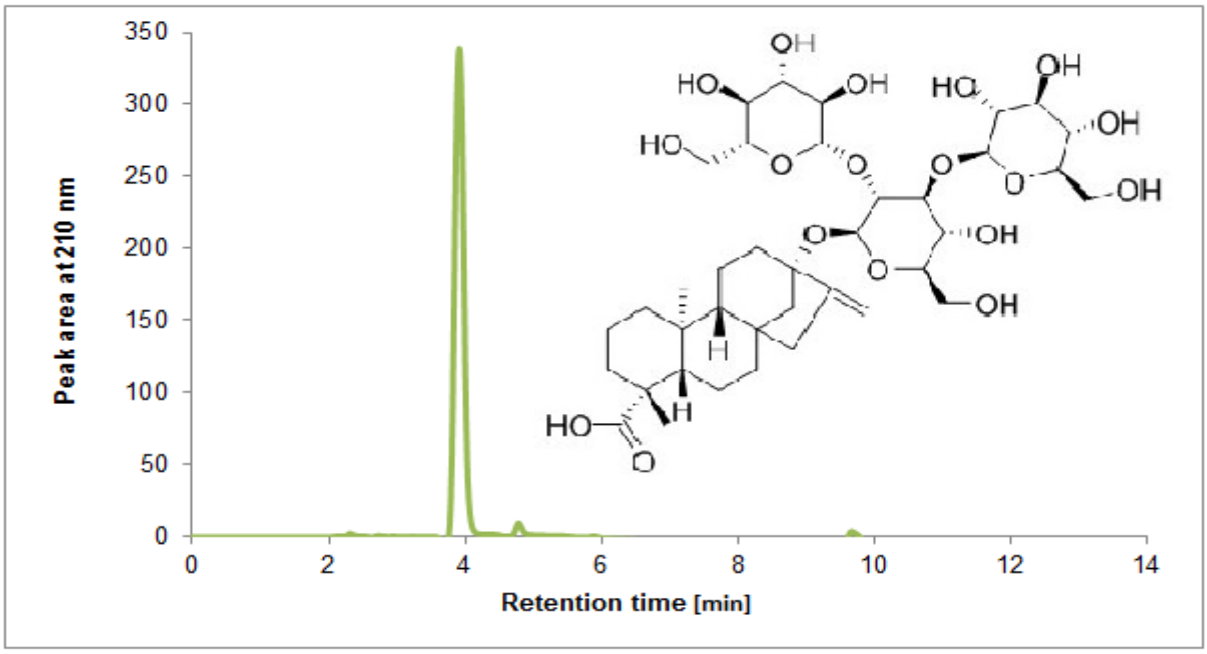


Figure 3.2 Chromatogram of rebaudioside B, under separation (75 % ACN, 25 % 10 mM $NaH_2PO_4 \times H_2O$ pH=3) at a flow rate of 1 ml/min. Injection volume was 20 µl, pressure 115 bar, temperature 25 °C and stop time 12 min. Column APS-2 HYPERSIL (150 × 4.6 mm + 50 × 4.6 mm); concentration 1 mg/ml

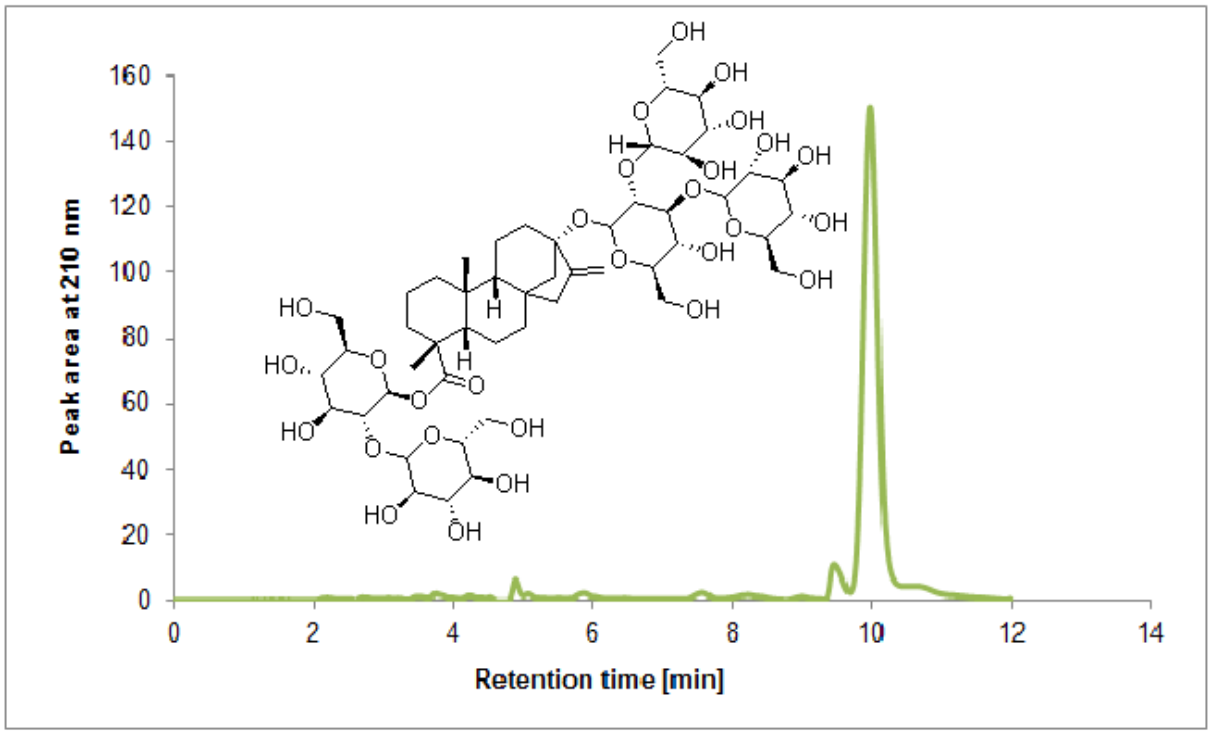


Figure 3.3 Chromatogram of rebaudioside D, under separation (75 % ACN, 25 % 10 mM $NaH_2PO_4 \times H_2O$ pH=3) at a flow rate of 1 ml/min. Injection volume was 20 µl, pressure 115 bar, temperature 25 °C and stop time 12 min. Column APS-2 HYPERSIL (150 × 4.6 mm + 50 × 4.6 mm); concentration 1 mg/ml

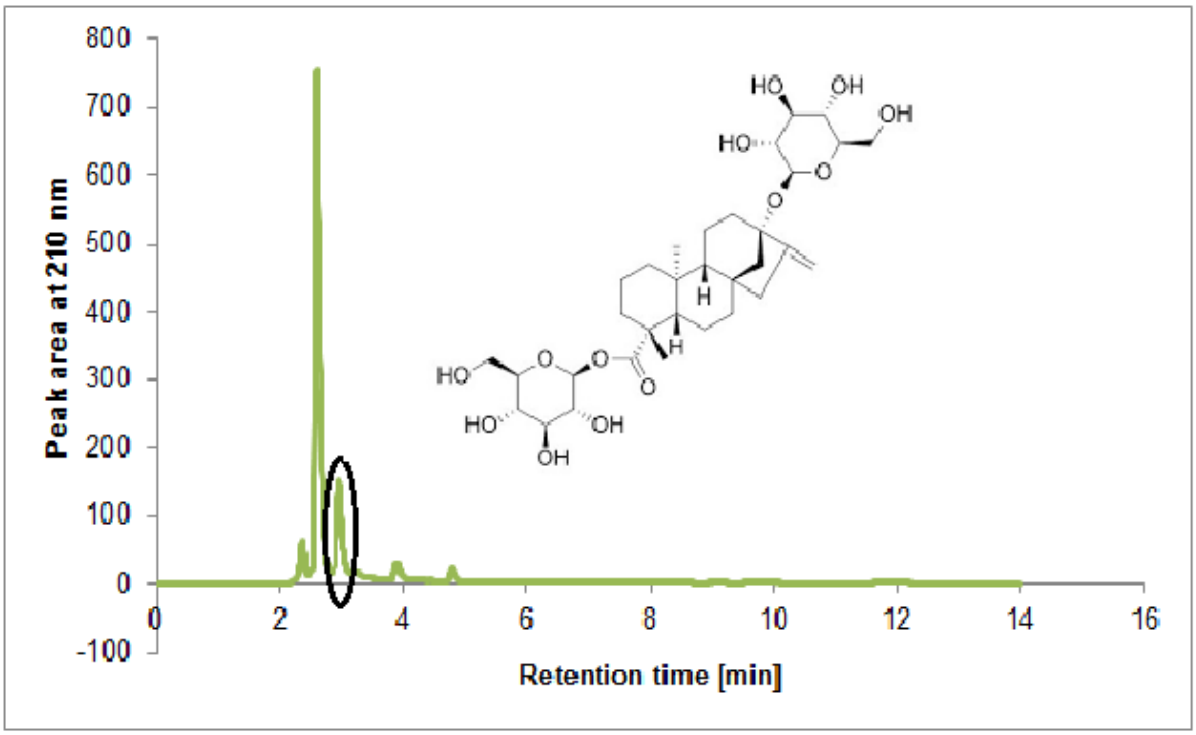


Figure 3.4 Chromatogram of rubusoside, under separation (75 % ACN, 25 % 10 mM $NaH_2PO_4 \times H_2O$ pH=3) at flow rate of 1 ml/min. Injection volume was 20 µl, pressure 115 bar, temperature 25 °C and stop time 12 min. Column APS-2 HYPERSIL (150 × 4.6 mm + 50 × 4.6 mm); concentration 1 mg/ml

3.1 Stability of steviosides in citric acid buffer at 6 °C (refrigerator temperature)

Figure 3.1.1 Rebaudioside A stability over one week incubation in citric acid buffer at different pH values at 6 °C

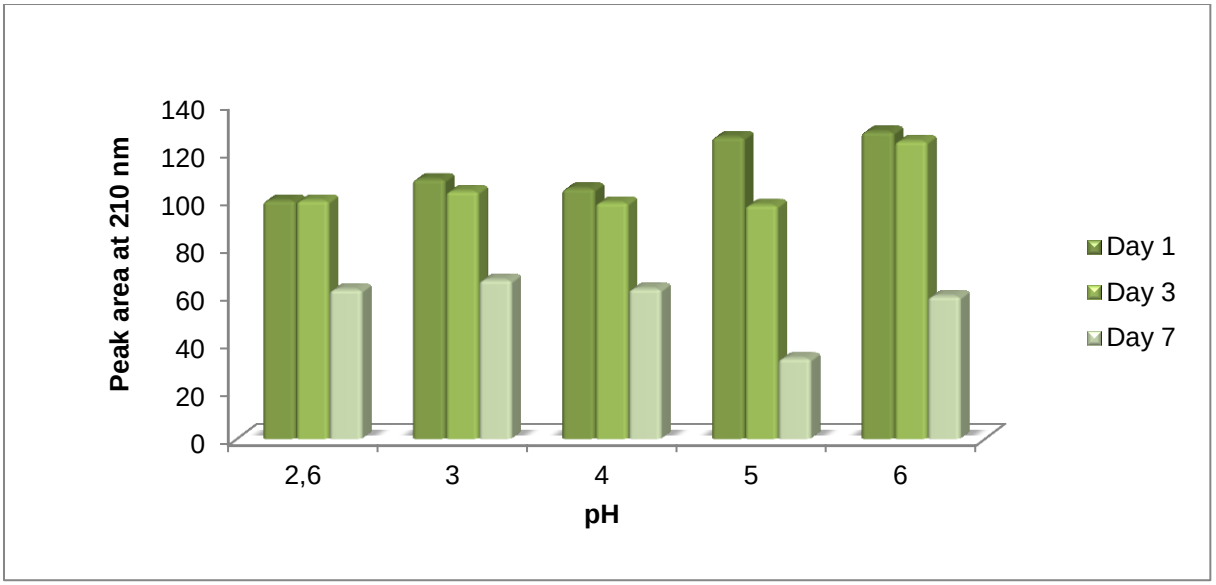


Figure 3.1.2 Rebaudioside B stability over one week incubation in citric acid buffer at different pH values at 6 °C

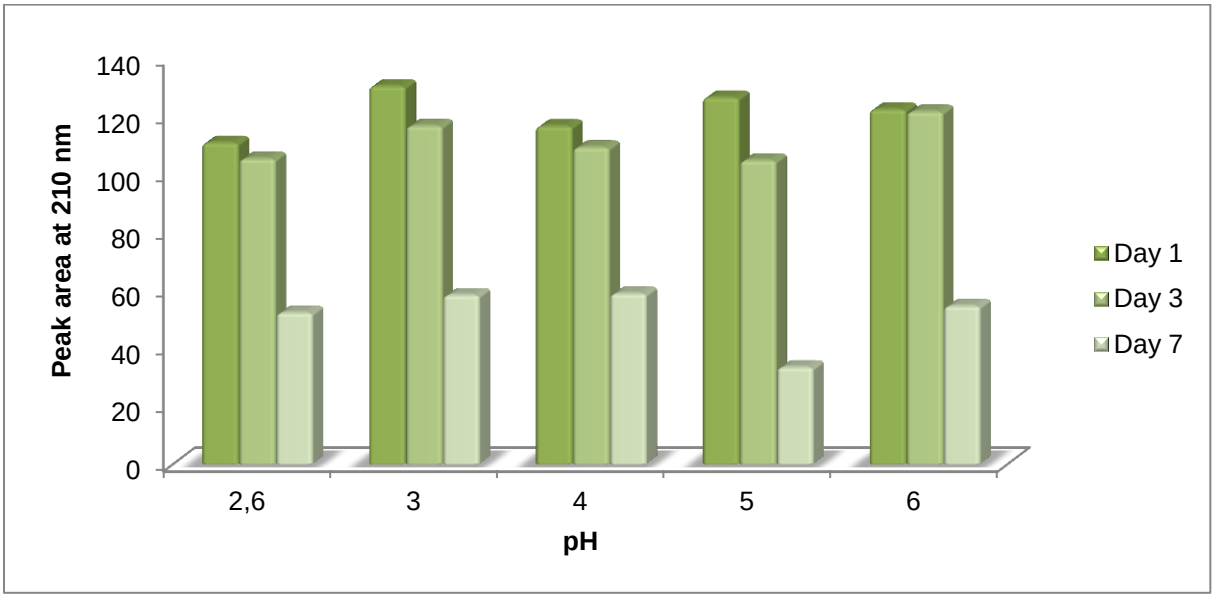


Figure 3.1.3 Rebaudioside D stability over one week incubation in citric acid buffer at different pH values at 6 °C

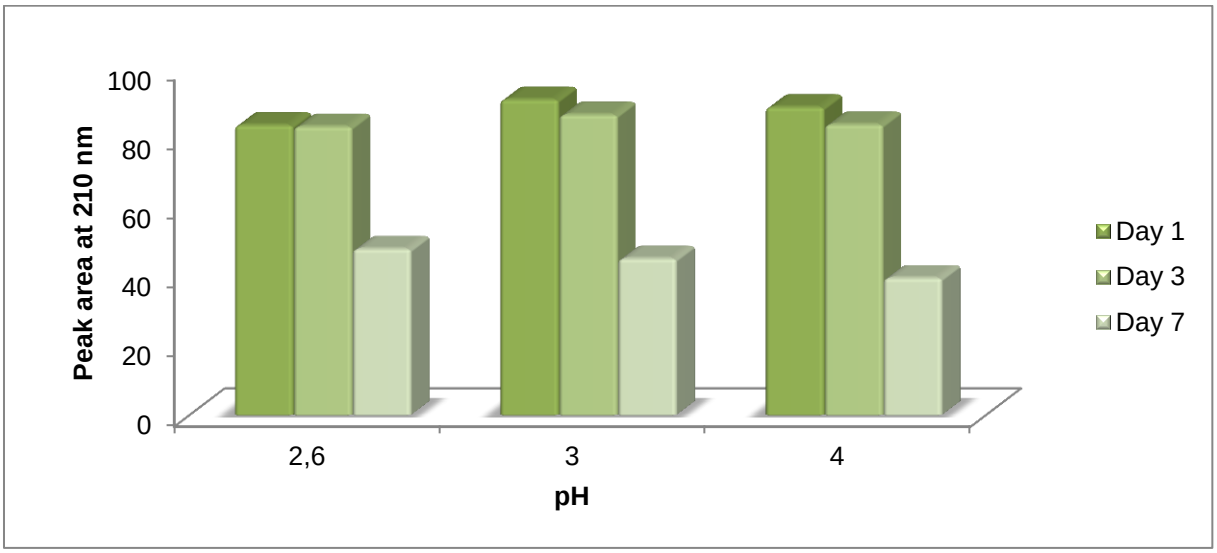
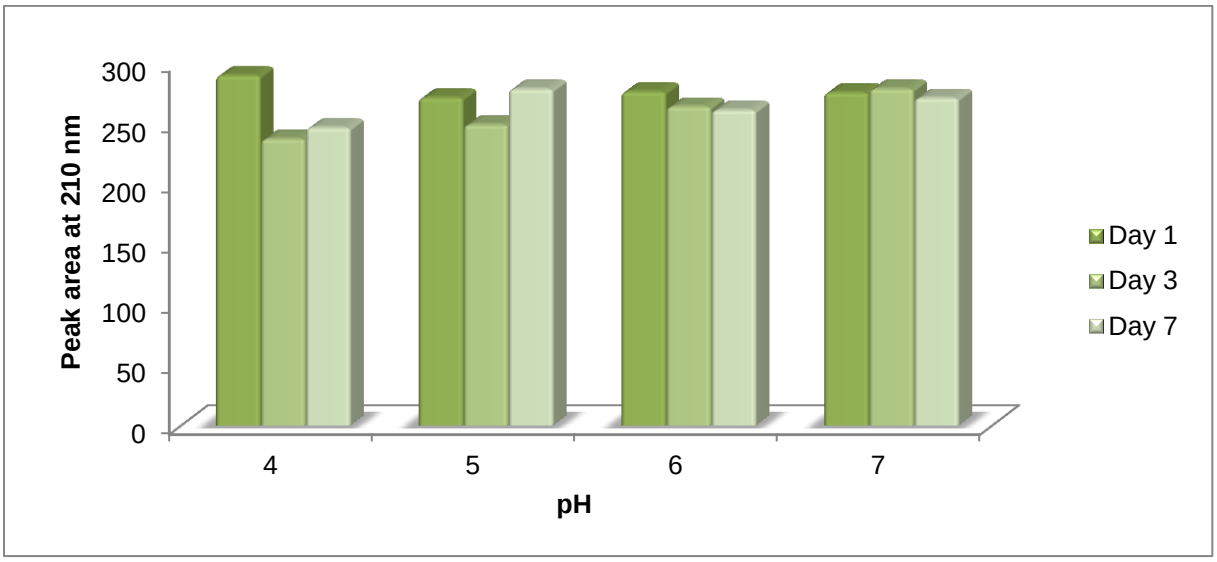


Figure 3.1.4 Rubusoside stability over one week incubation in citric acid buffer at different pH values at 6 °C



3.2 Stability of steviosides in citric acid buffer at 23 °C (room temperature)

Figure 3.2.1 Rebaudioside A stability over one week incubation in citric acid buffer at different pH values at 23 °C

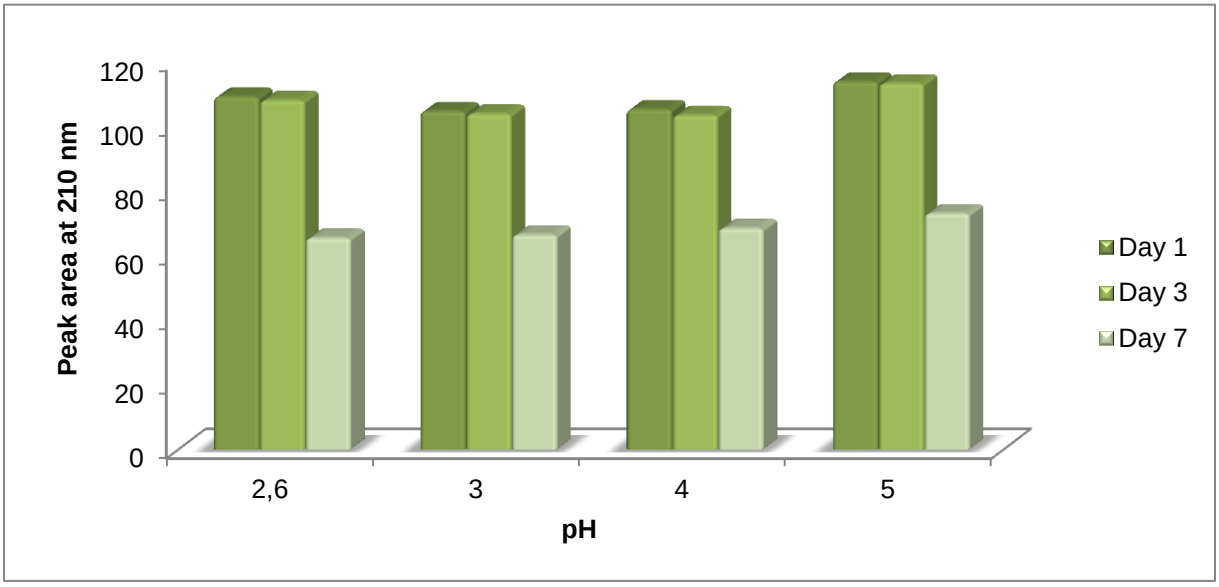


Figure 3.2.2 Rebaudioside B stability over one week incubation in citric acid buffer at different pH values at 23 °C

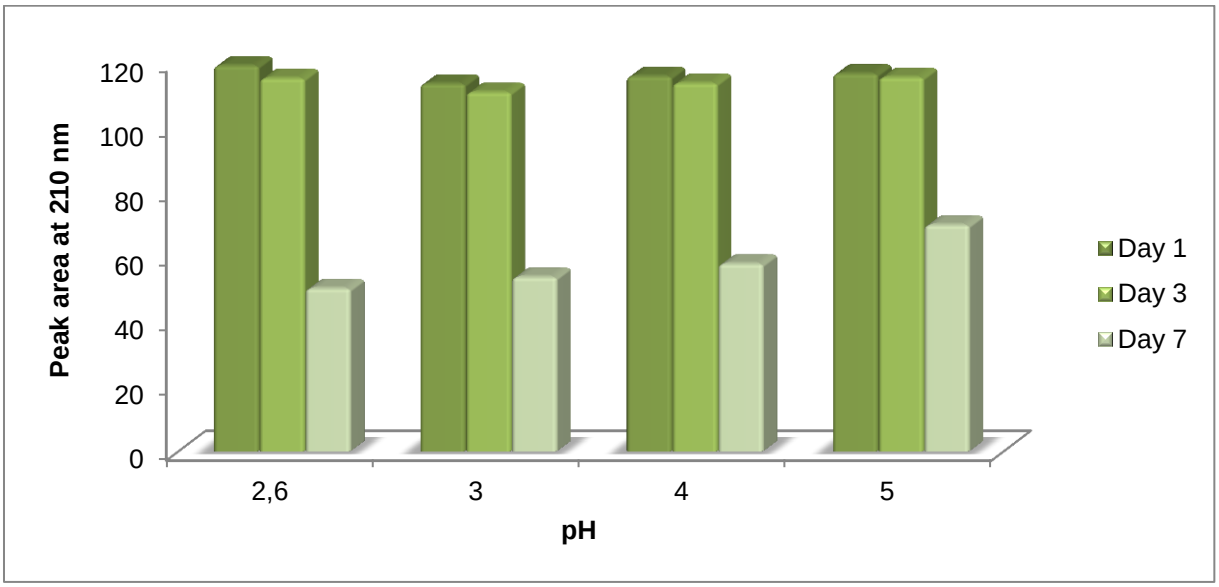


Figure 3.2.3 Rebaudioside D stability over one week incubation in citric acid buffer at different pH values at 23 °C

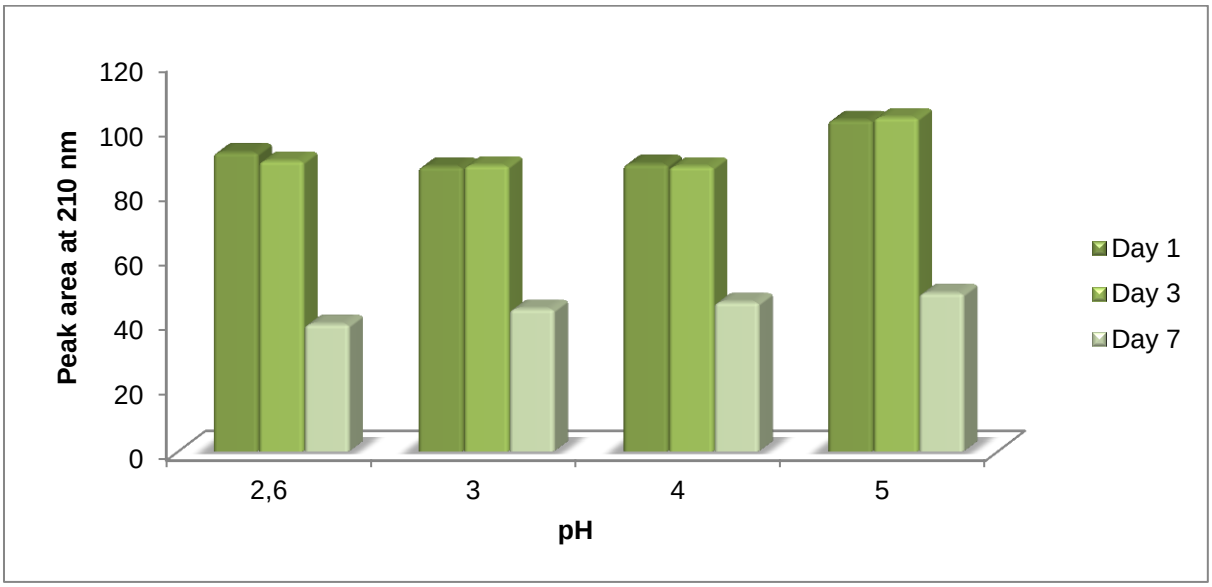
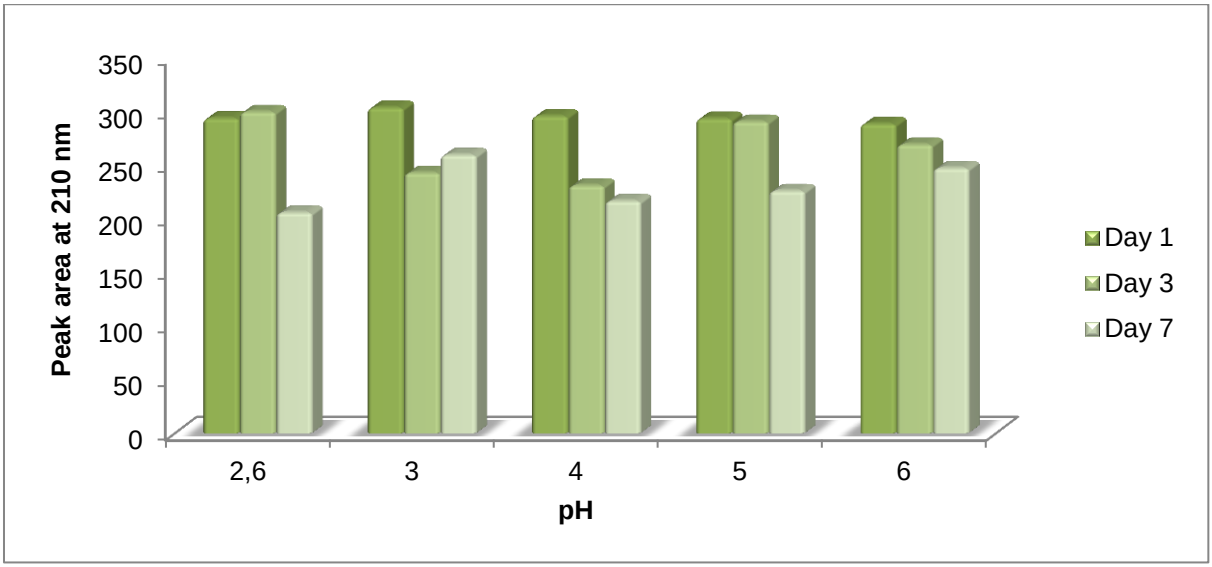


Figure 3.2.4 Rubusoside stability over one week incubation in citric acid buffer at different pH values at 23 °C



3.3 Stability of steviosides in phosphoric acid buffer at 6 °C (refrigerator temperature)

Figure 3.3.1 Rebaudioside A stability over one month incubation in phosphoric acid buffer at different pH values at 6 °C

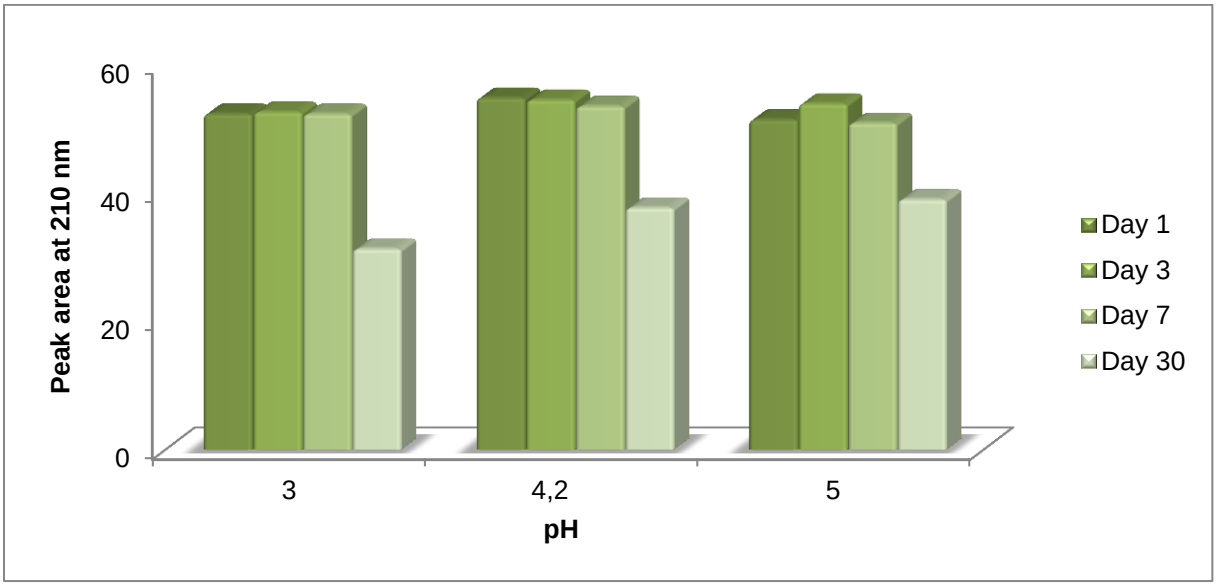


Figure 3.3.2 Rebaudioside B stability over one month incubation in phosphoric acid buffer at different pH values at 6 °C

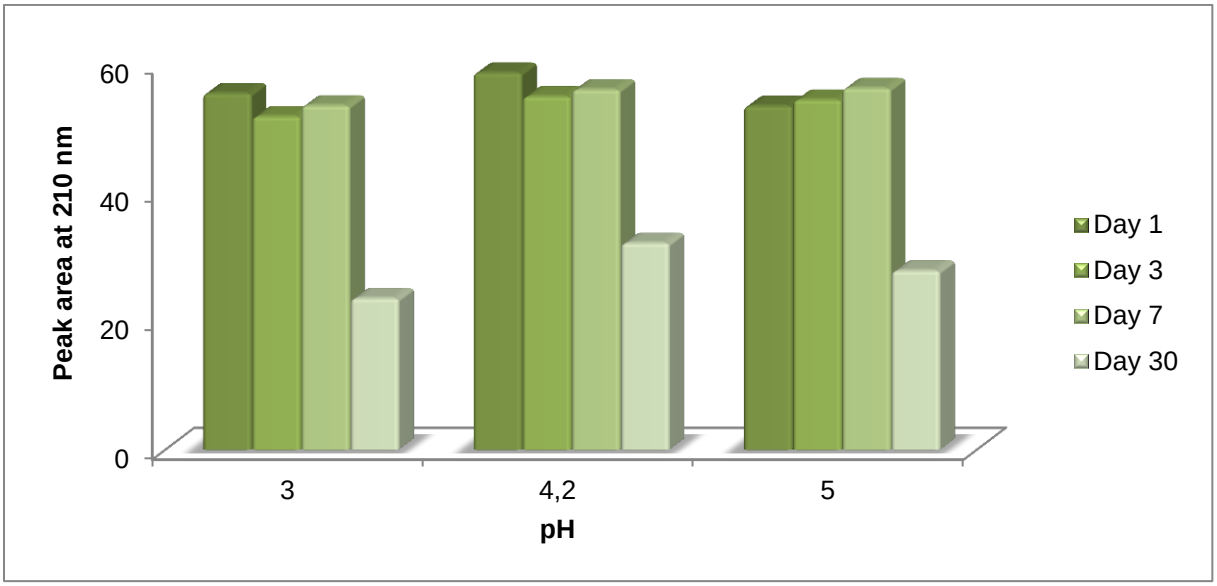


Figure 3.3.3 Rebaudioside D stability over one month incubation in phosphoric acid buffer at different pH values at 6 °C

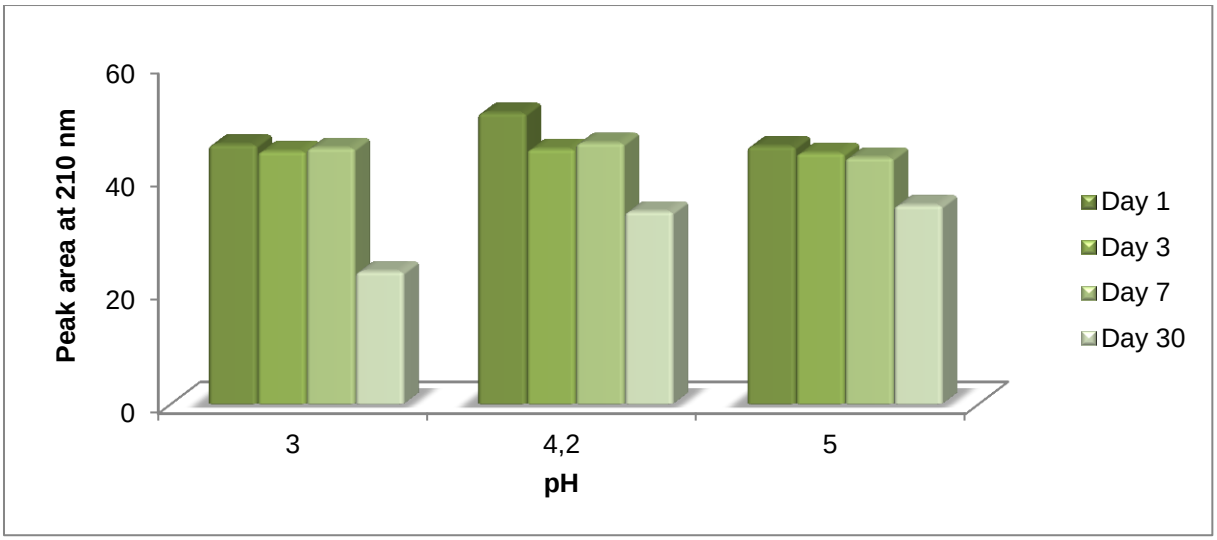
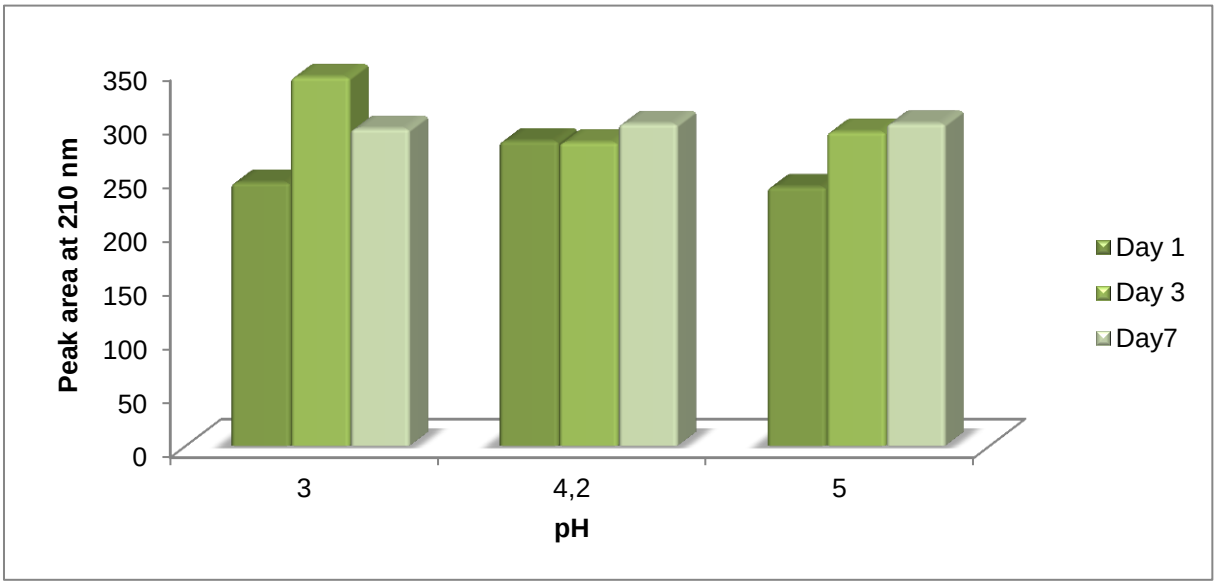


Figure 3.3.4 Rubusoside stability over one week incubation in phosphoric acid buffer at different pH values at 6 °C



3.4 Stability of steviosides in phosphoric acid buffer at 23 °C (room temperature)

Figure 3.4.1 Rebaudioside A stability over one month incubation in phosphoric acid buffer at different pH values at 23 °C

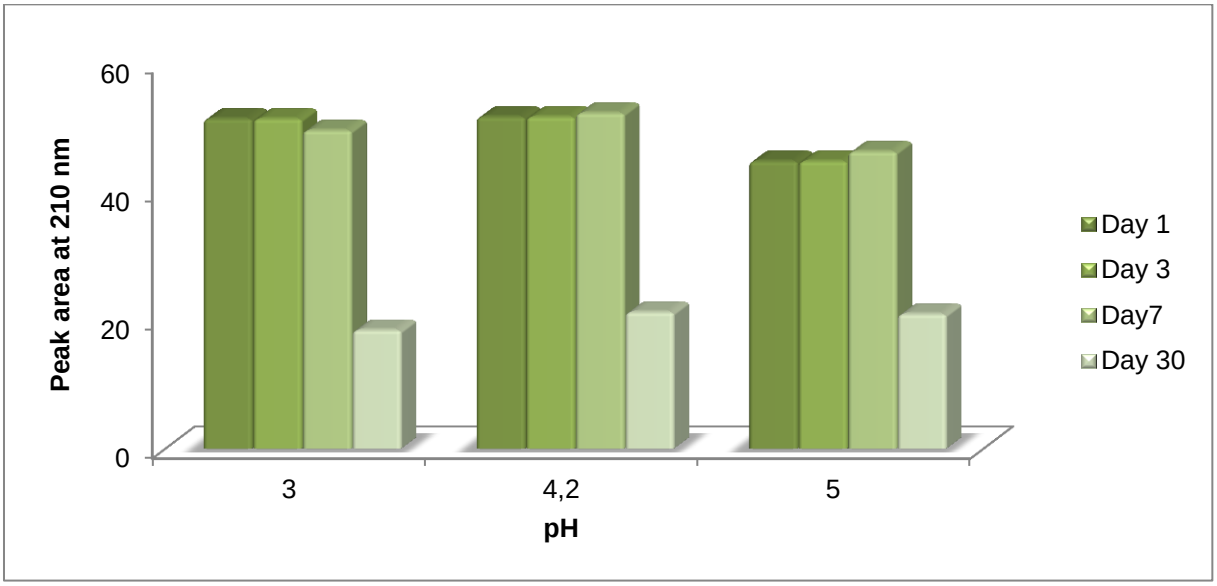


Figure 3.4.2 Rebaudioside B stability over one month incubation in phosphoric acid buffer at different pH values at 23 °C

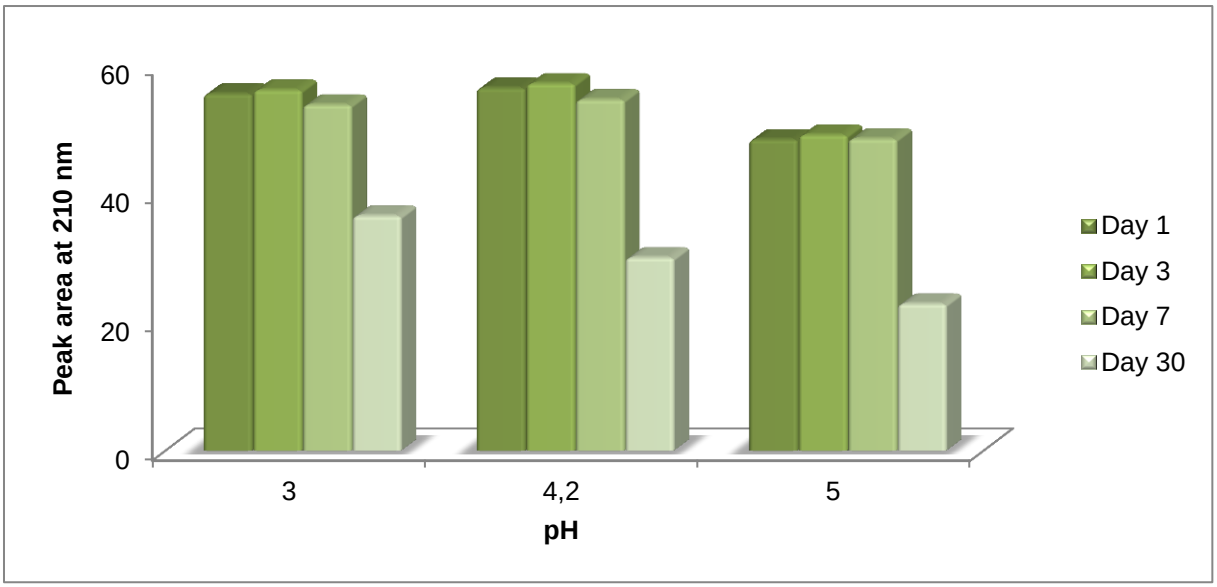


Figure 3.4.3 Rebaudioside D stability over one month incubation in phosphoric acid buffer at different pH values at 23 °C

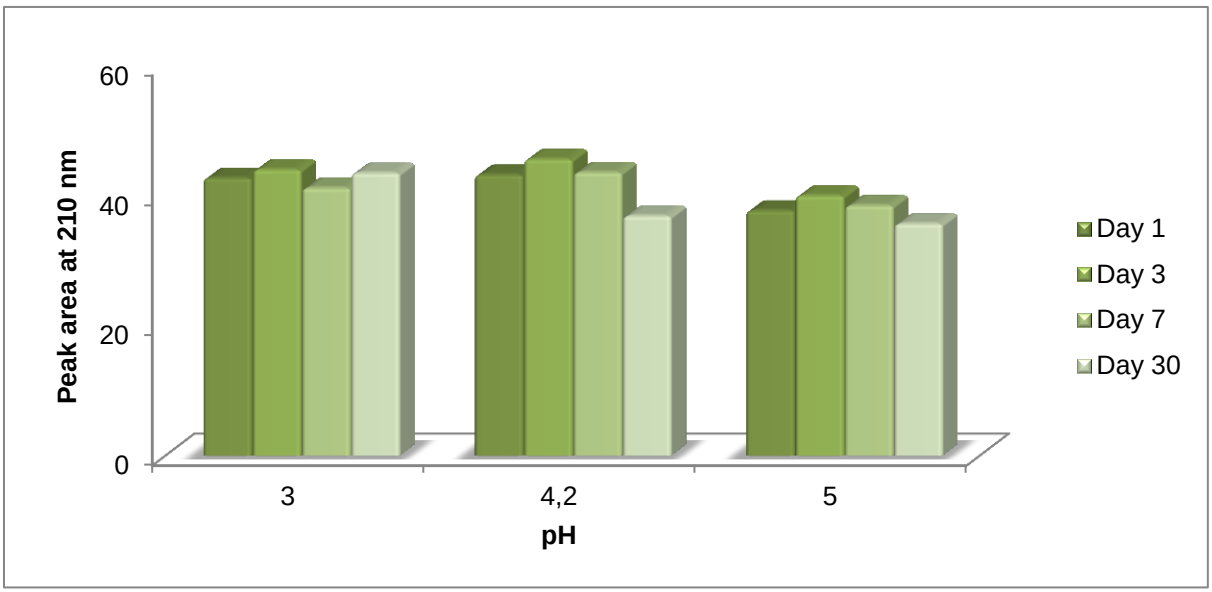
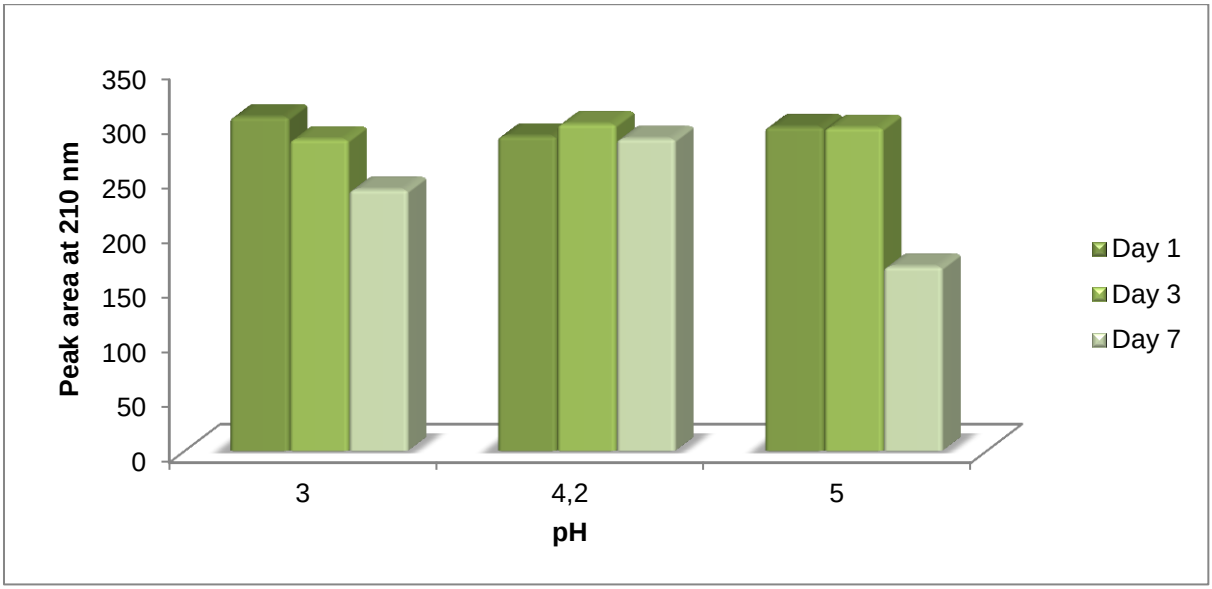


Figure 3.4.4 Rubusoside stability over one week incubation in phosphoric acid buffer at different pH values at 23 °C



3.5 Stability of steviosides in citric acid buffer at elevated temperatures

Figure 3.5.1 Rebaudioside A stability incubation in citric acid buffer pH=3

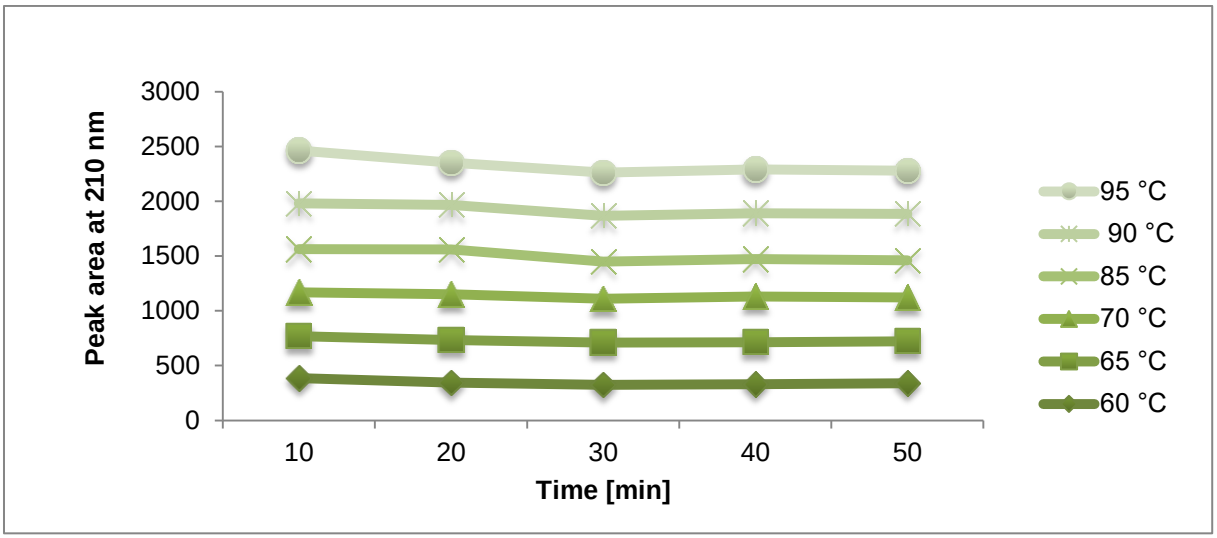


Figure 3.5.2 Rebaudioside A stability incubation in citric acid buffer pH=4

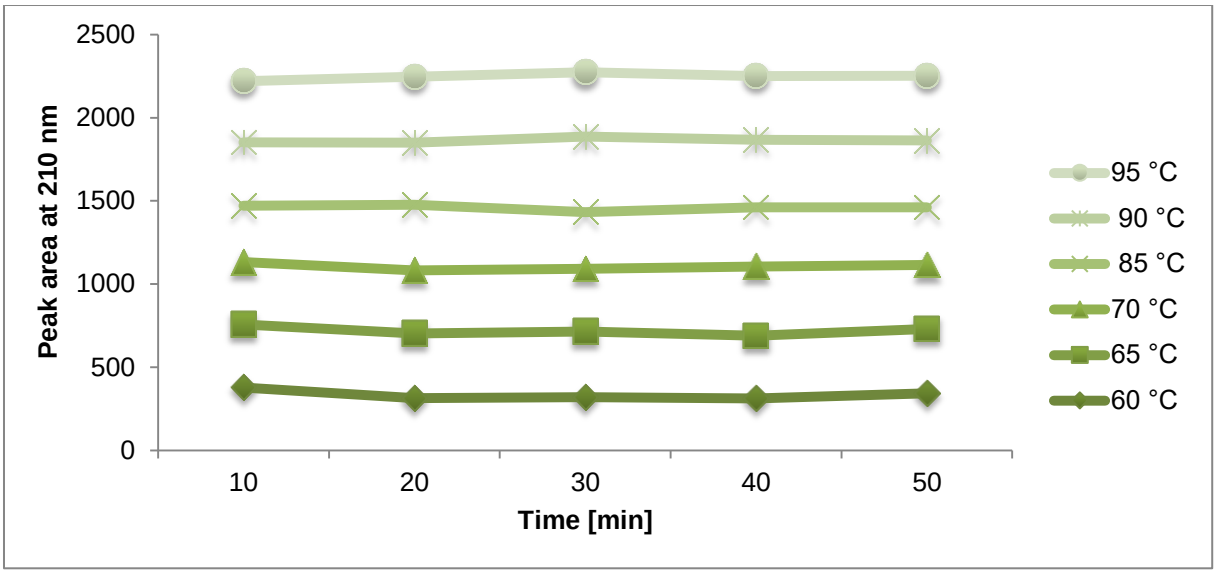


Figure 3.5.3 Rebaudioside A stability incubation in citric acid buffer pH=5

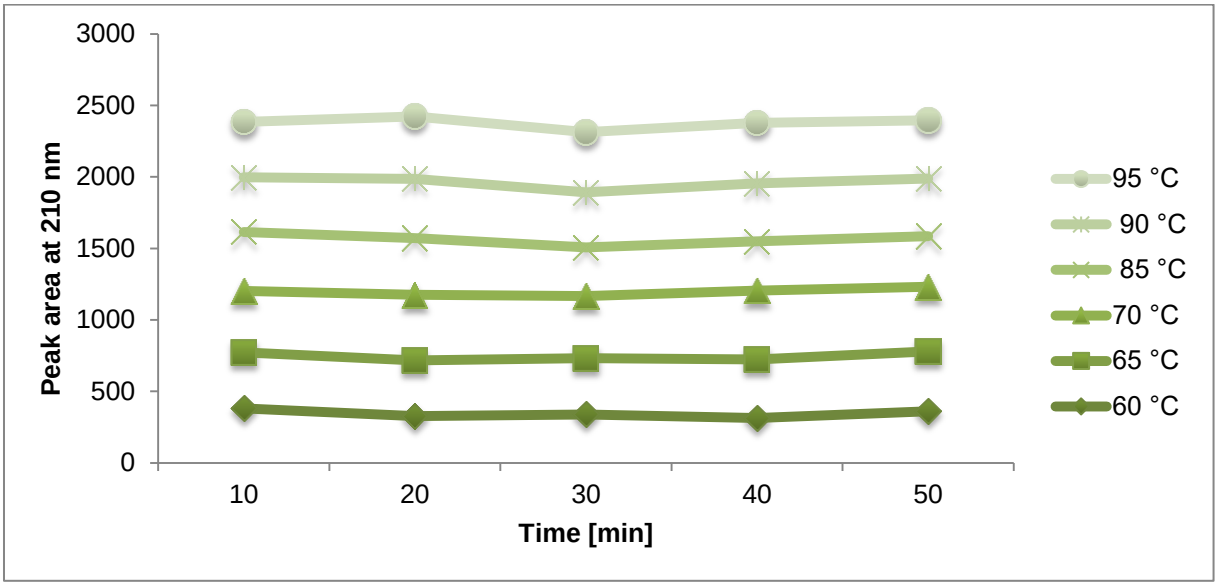


Figure 3.5.4 Rebaudioside B stability incubation in citric acid buffer pH=3

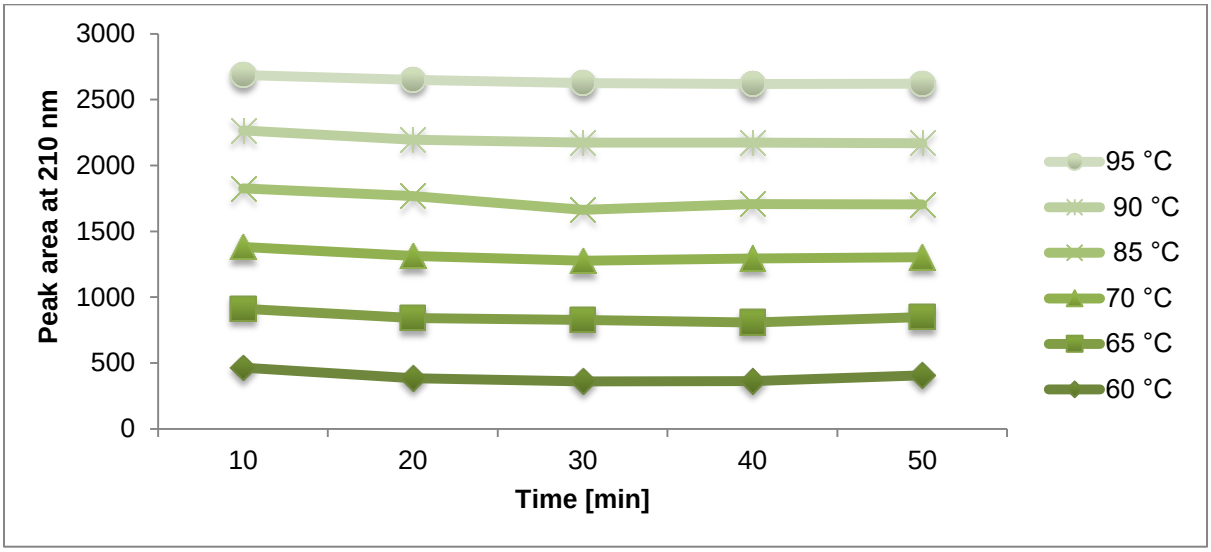


Figure 3.5.5 Rebaudioside B stability incubation in citric acid buffer pH=4

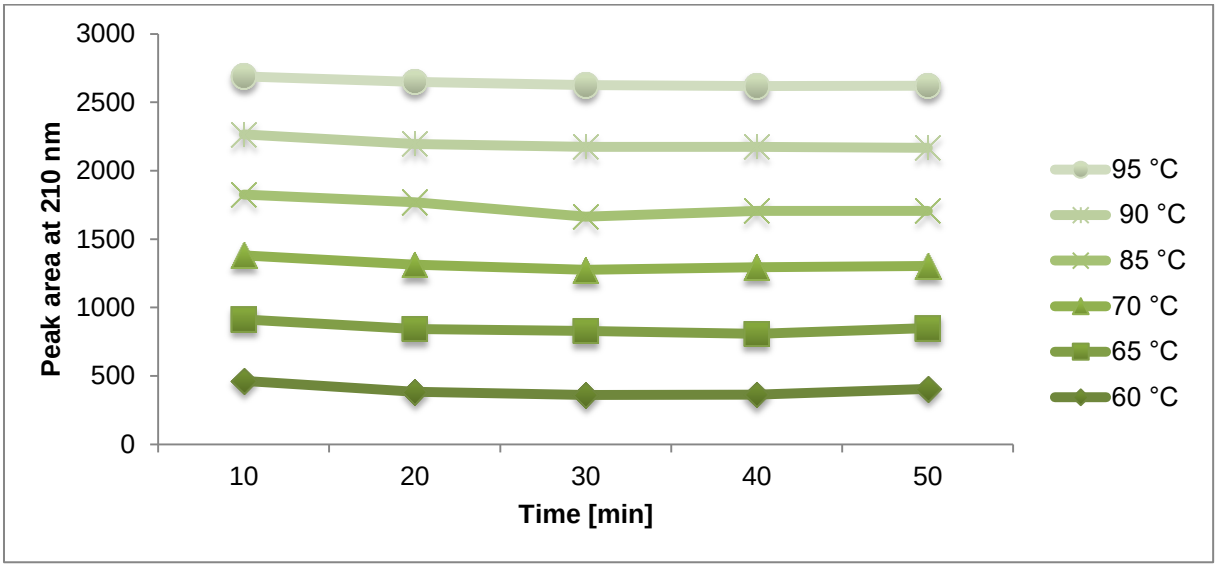


Figure 3.5.6 Rebaudioside B stability incubation in citric acid buffer pH=5

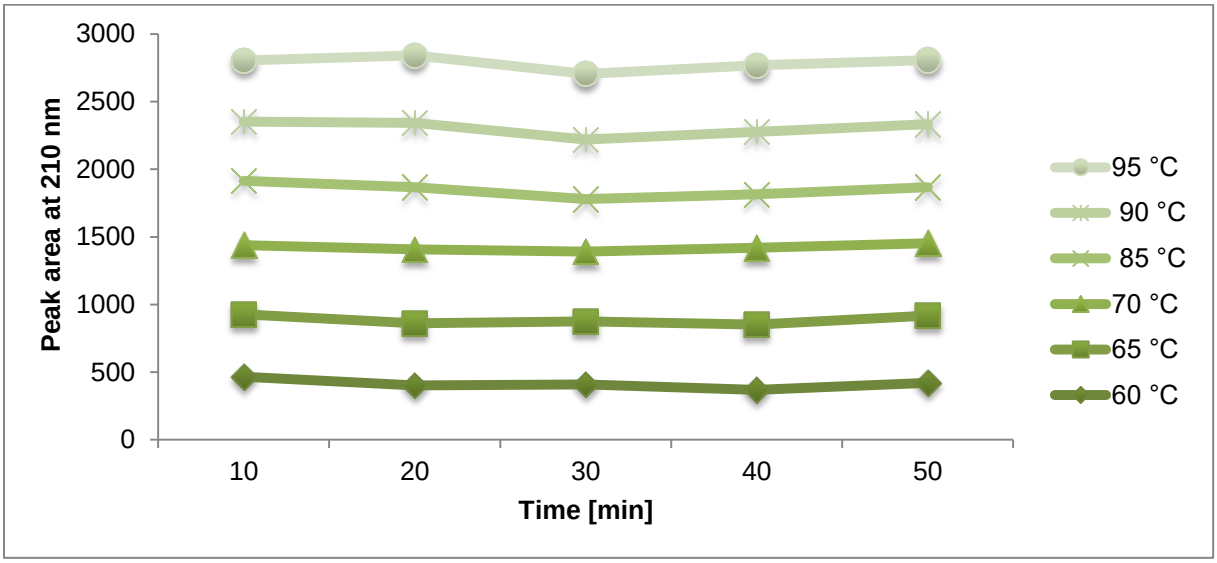


Figure 3.5.7 Rebaudioside D stability incubation in citric acid buffer pH=3

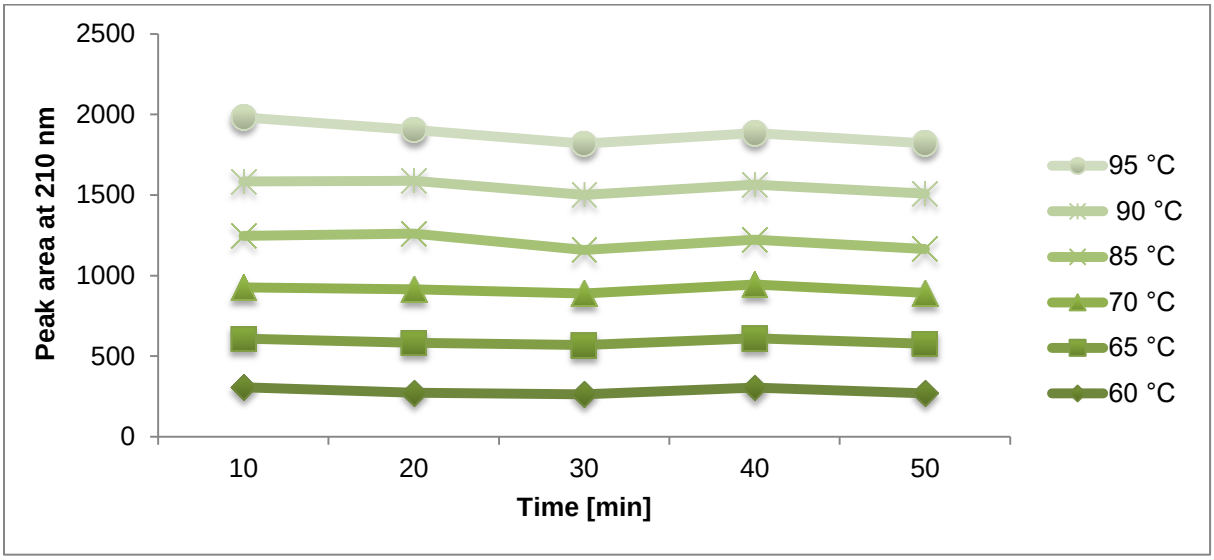


Figure 3.5.8 Rebaudioside D stability incubation in citric acid buffer pH=4

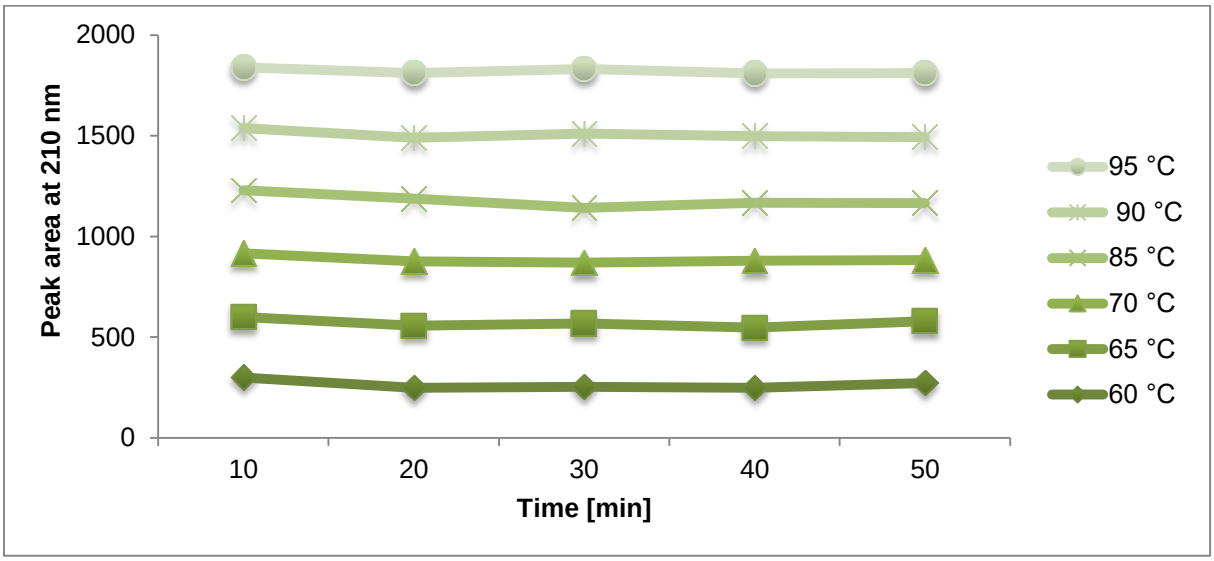
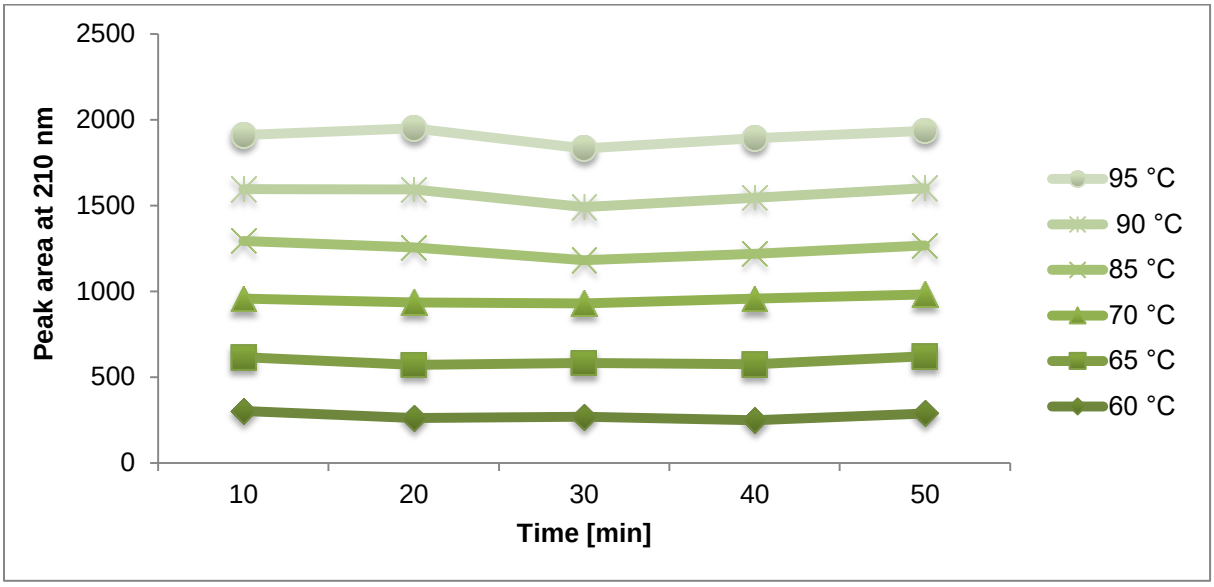


Figure 3.5.9 Rebaudioside D stability incubation in citric acid buffer pH=5



3.6 Stability of steviosides in phosphoric acid buffer at elevated temperatures

Figure 3.6.1 Rebaudioside A stability incubation in phosphoric acid buffer pH=3

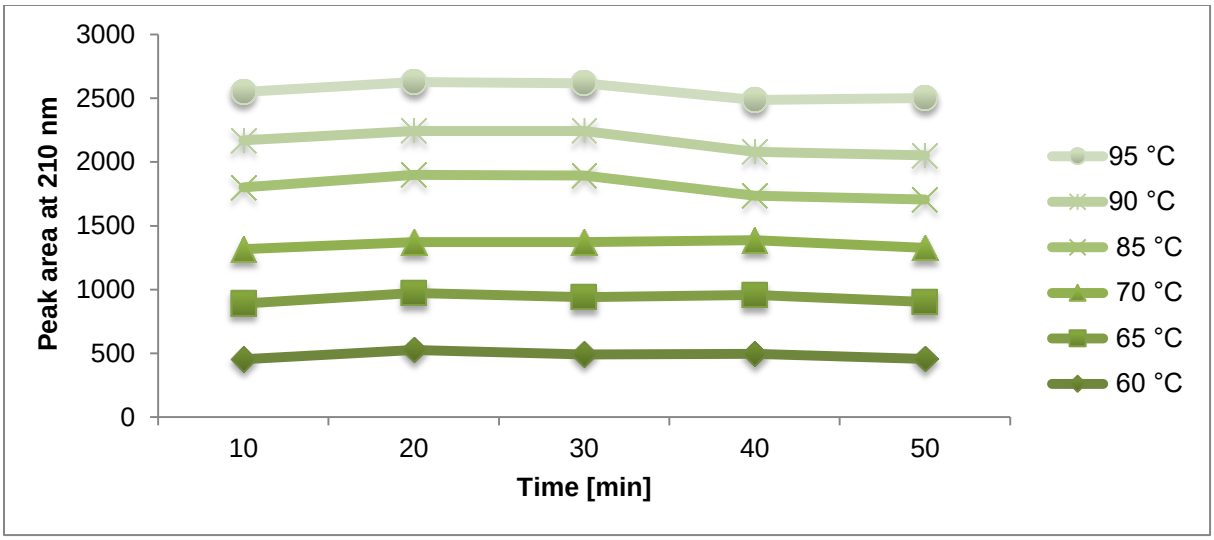


Figure 3.6.2 Rebaudioside A stability incubation in phosphoric acid buffer pH=4.2

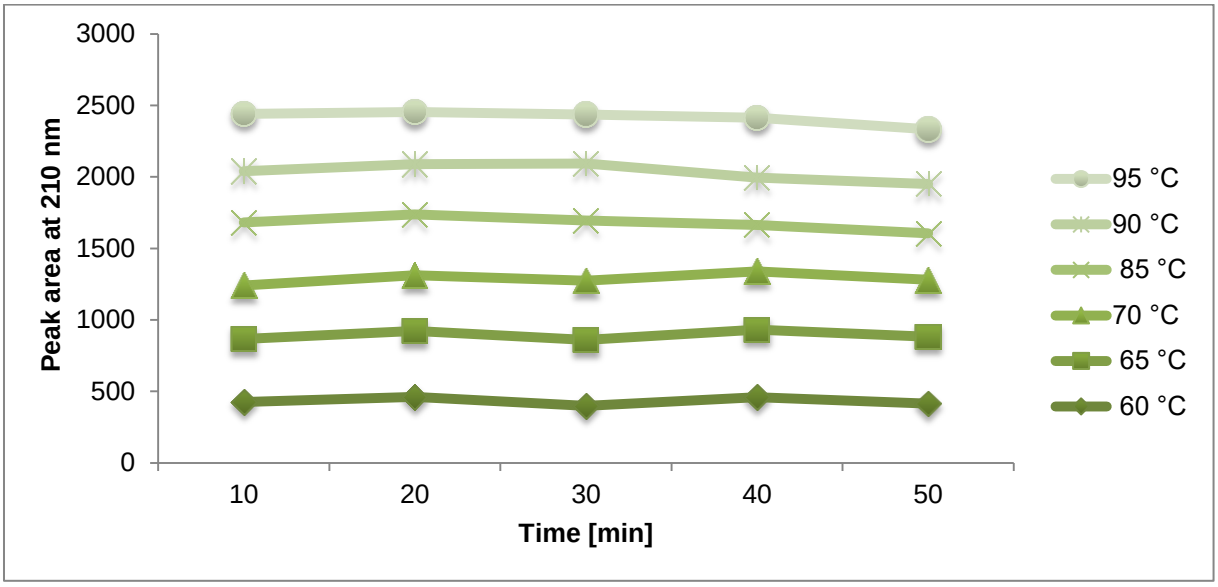


Figure 3.6.3 Rebaudioside A stability incubation in phosphoric acid buffer pH=5

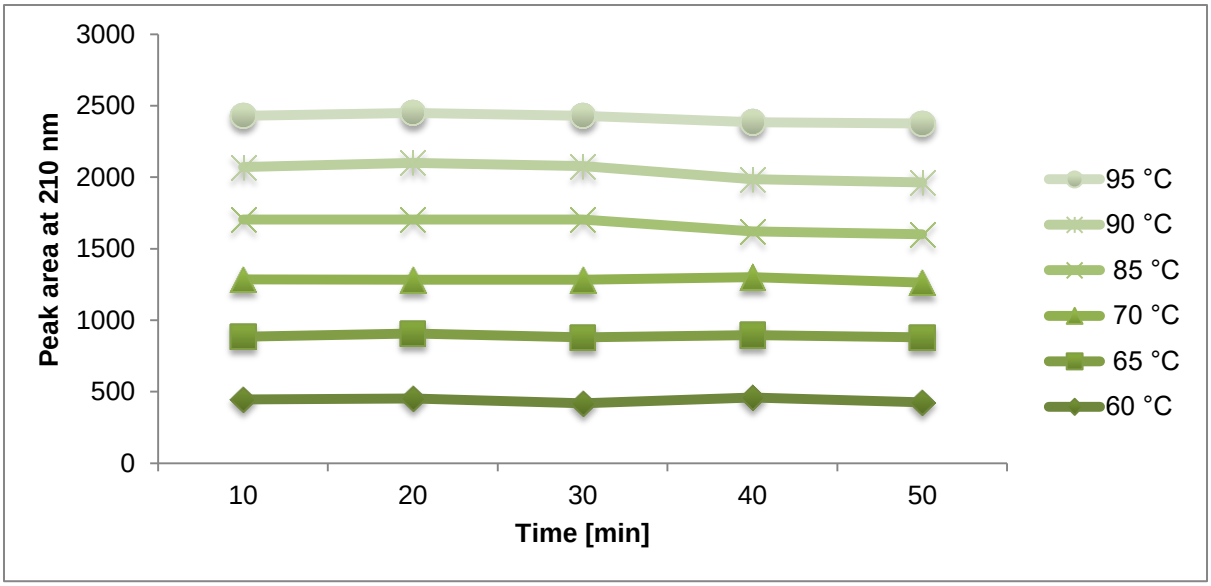


Figure 3.6.3 Rebaudioside A stability incubation in phosphoric acid buffer pH=5

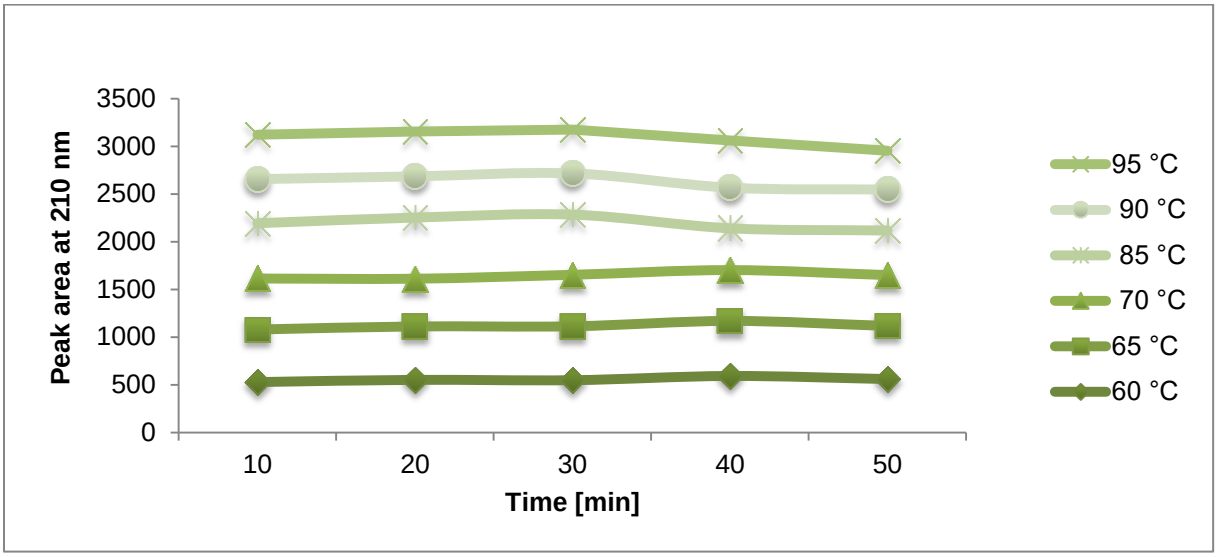


Figure 3.6.5 Rebaudioside B stability incubation in phosphoric acid buffer pH=4.2

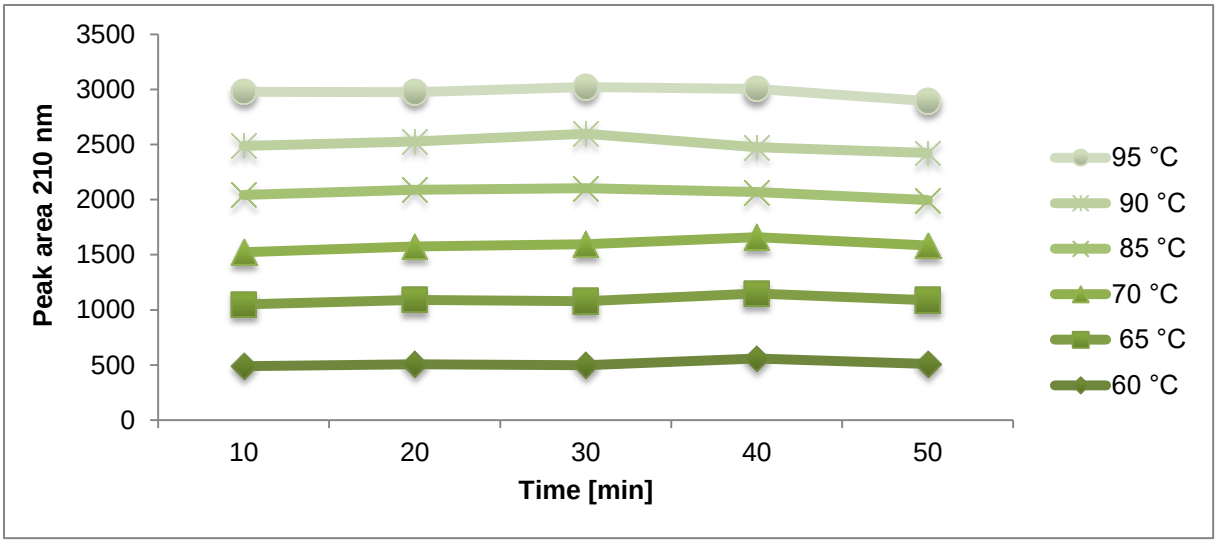


Figure 3.6.6 Rebaudioside B stability incubation in phosphoric acid buffer pH=5

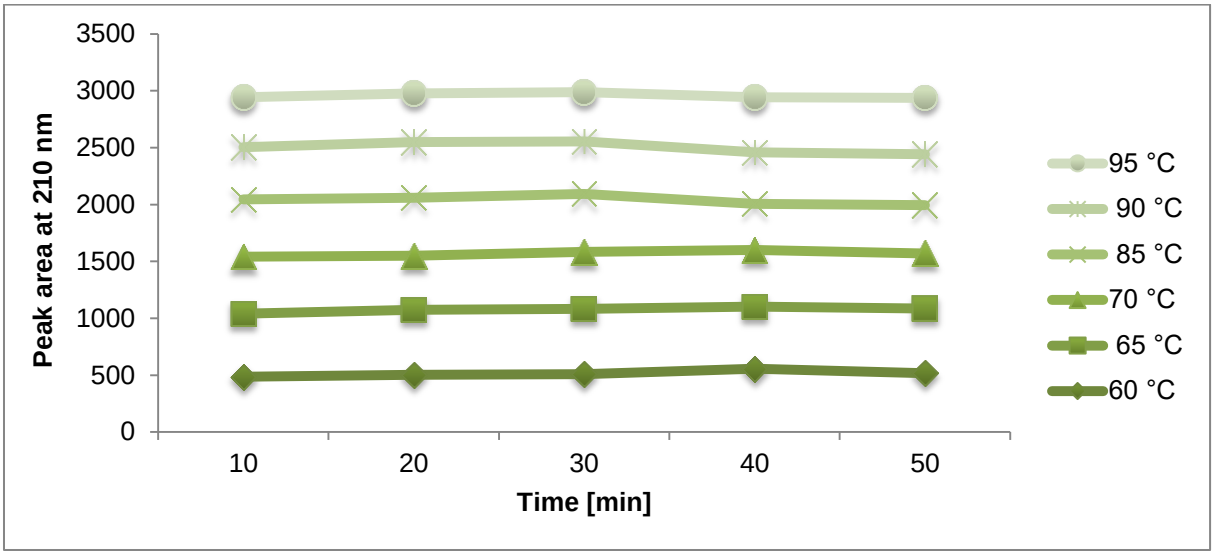


Figure 3.6.7 Rebaudioside D stability incubation in phosphoric acid buffer pH=3

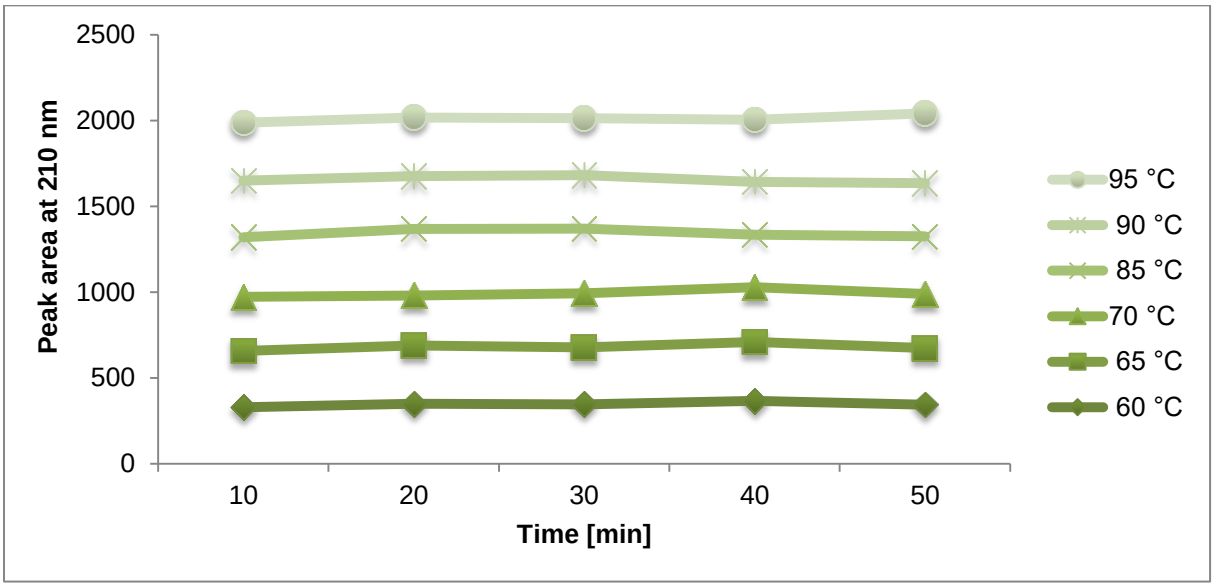


Figure 3.6.8 Rebaudioside D stability incubation in phosphoric acid buffer pH=4.2

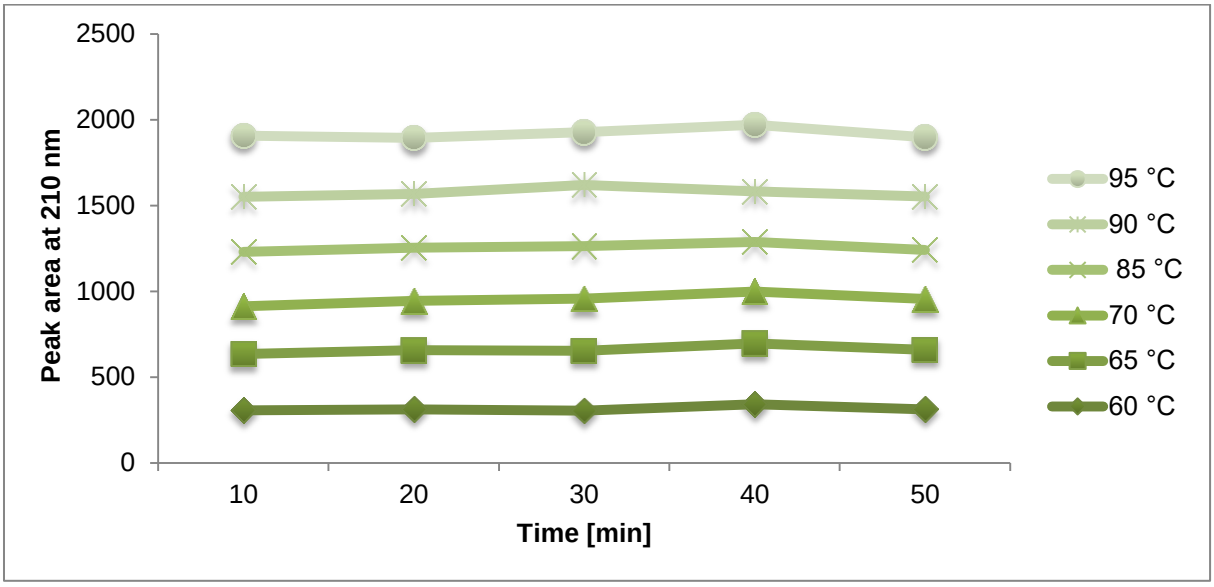
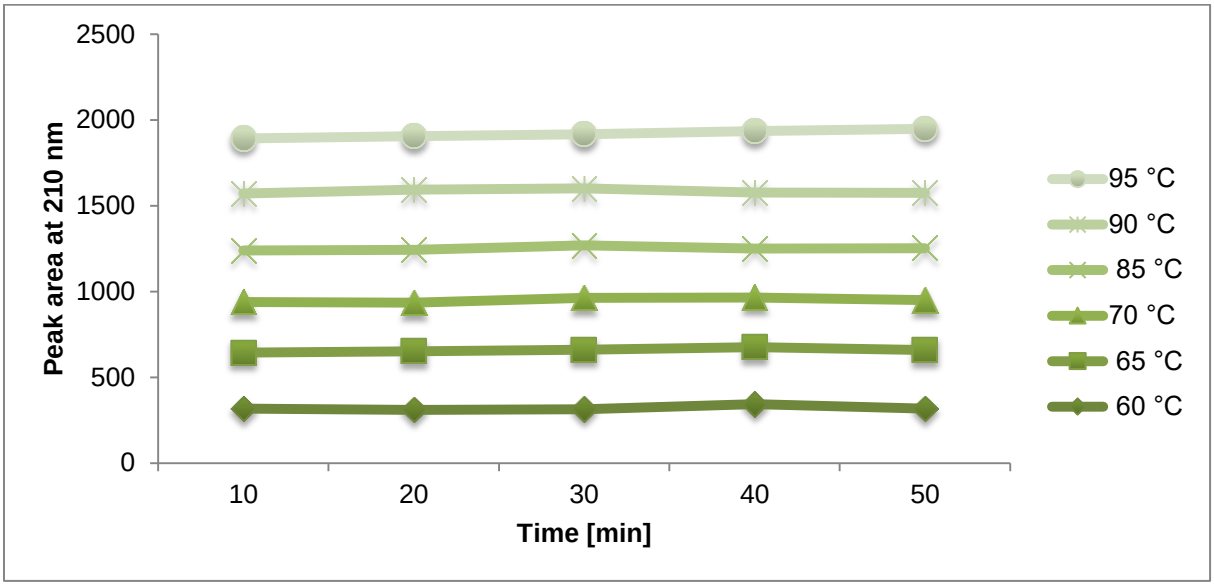


Figure 3.6.9 Rebaudioside D stability incubation in phosphoric acid buffer pH=5



3.7 Rubusoside stability in citric acid buffer at 72 °C

Figure 3.7.1 Rubusoside stability in citric acid buffer pH=2.6

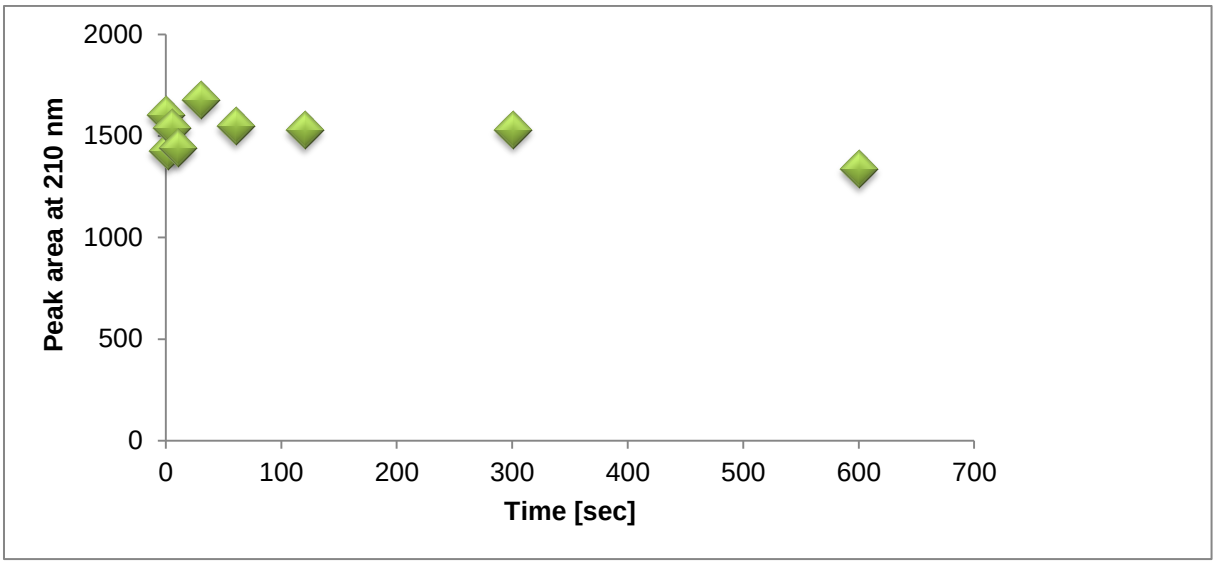


Figure 3.7.2 Rubusoside stability in citric acid buffer pH=3

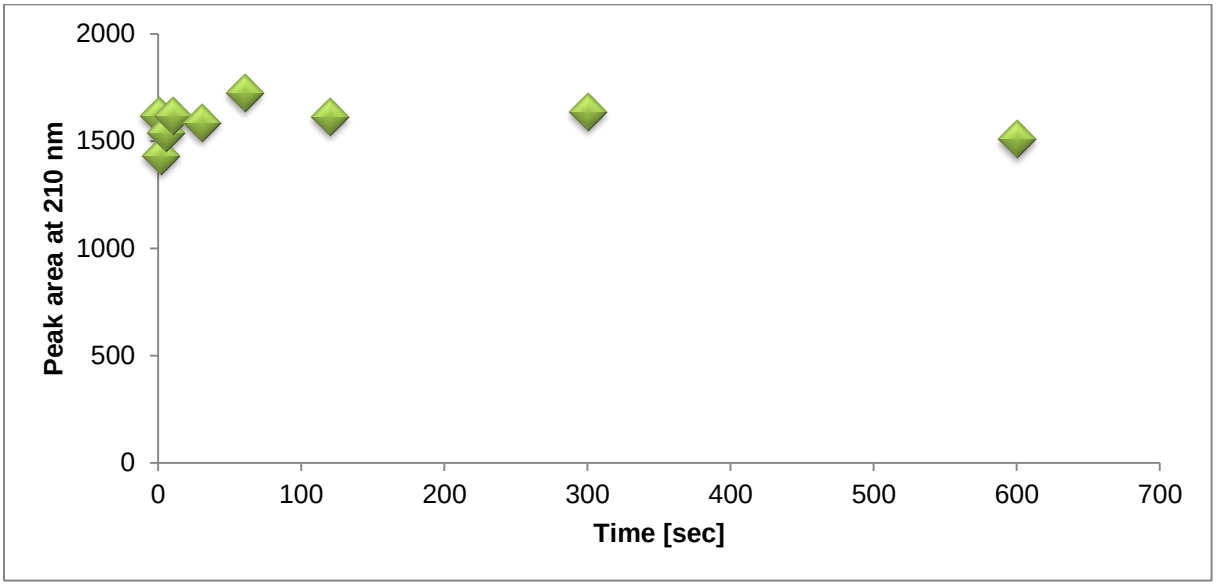


Figure 3.7.3 Rubusoside stability in citric acid buffer pH=4

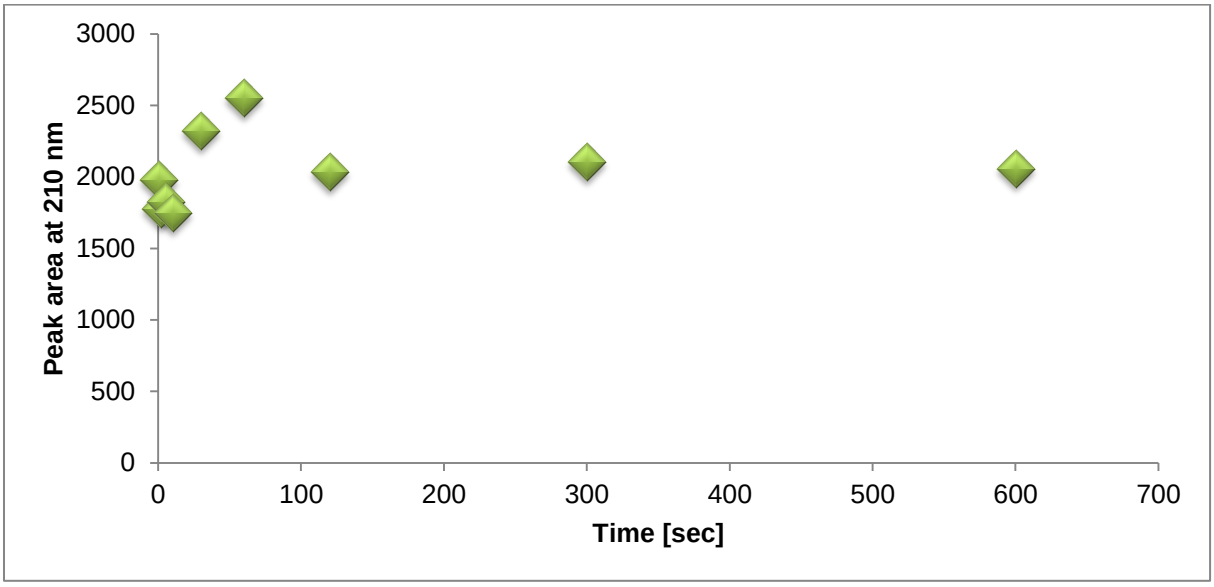


Figure 3.7.4 Rubusoside stability in citric acid buffer pH=5

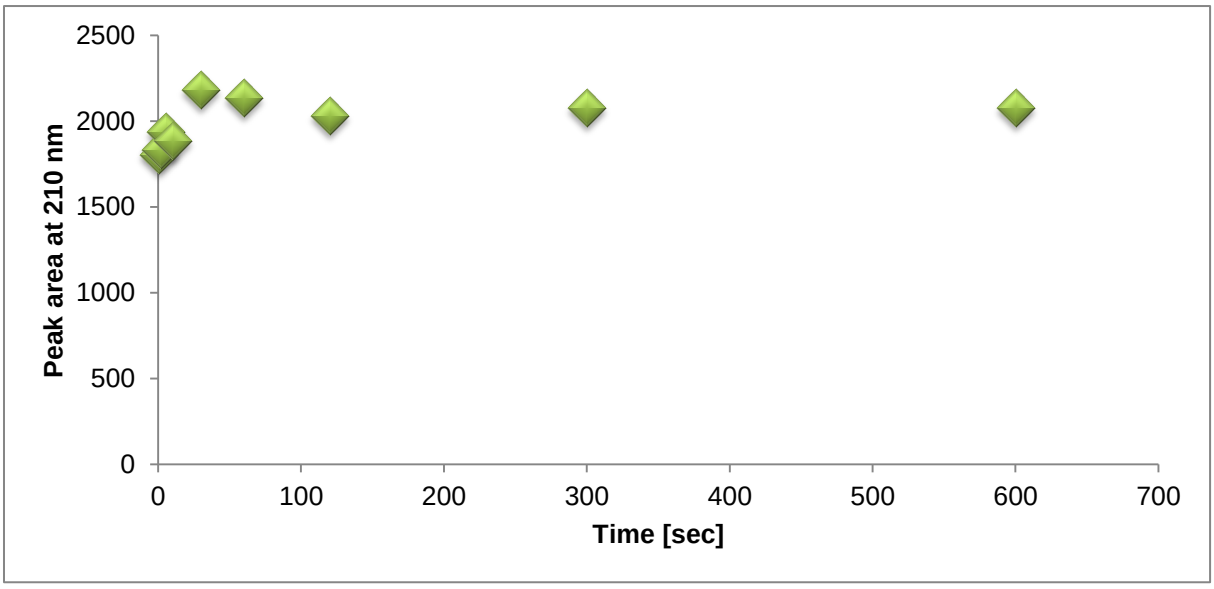
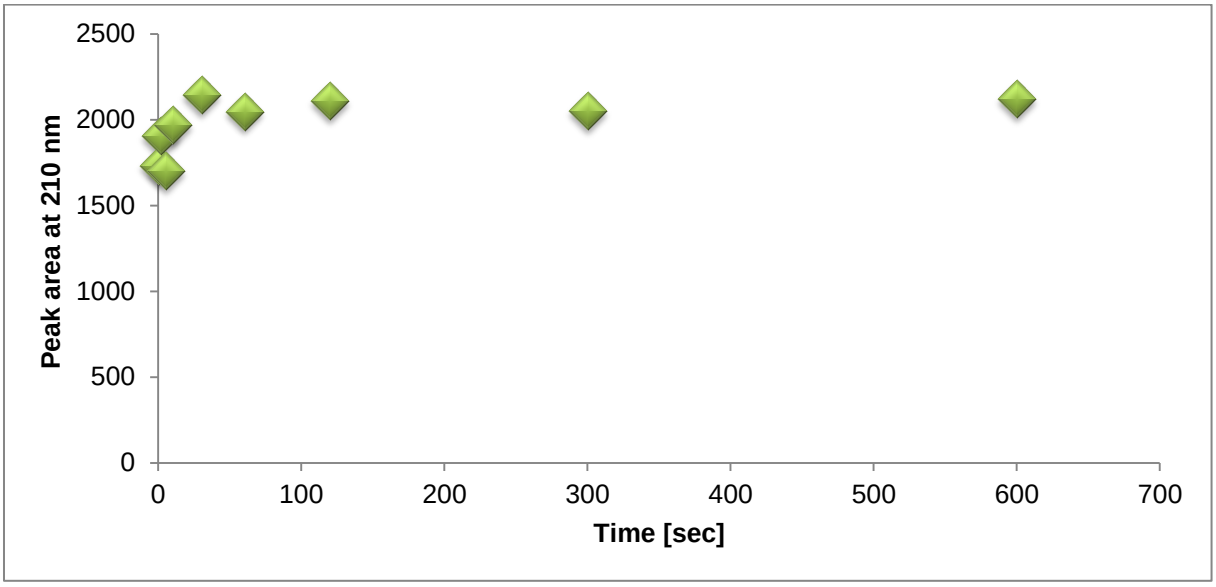


Figure 3.7.5 Rubusoside stability in citric acid buffer pH=6



3.8. Rubusoside stability in phosphoric acid buffer at 72 °C

Figure 3.8.1 Rubusoside stability in phosphoric acid buffer pH=3

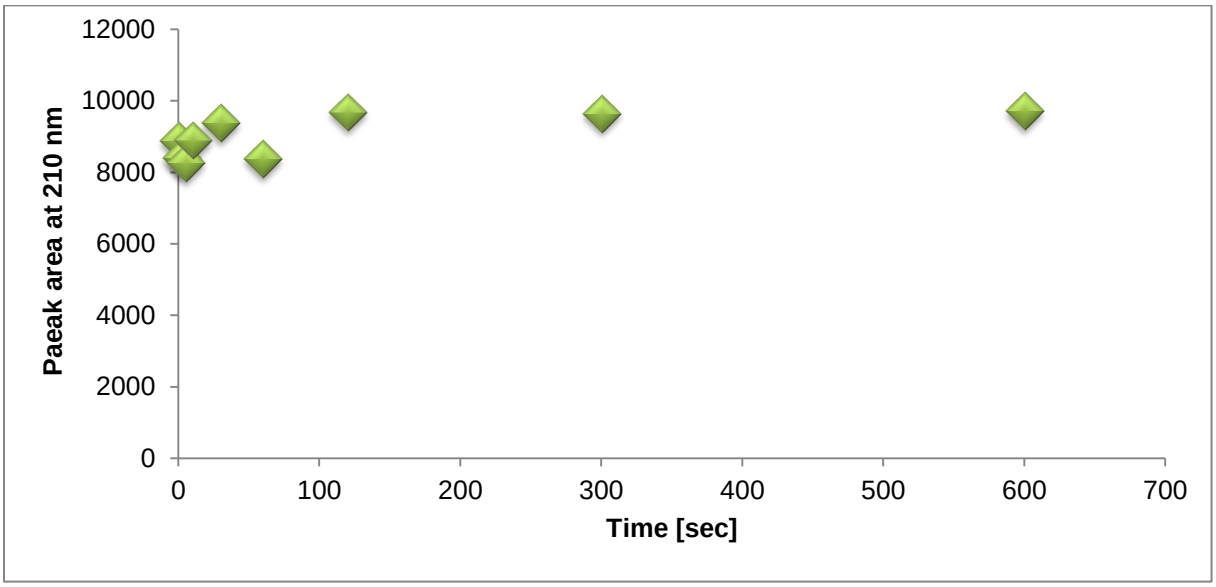


Figure 3.8.2 Rubusoside stability in phosphoric acid buffer pH=4.2

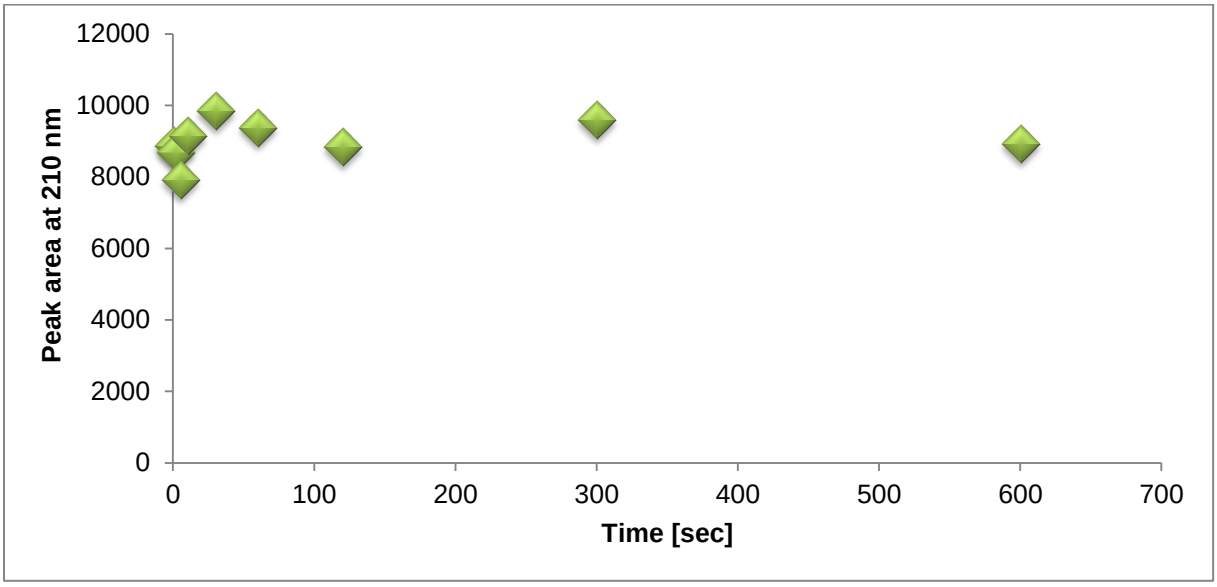
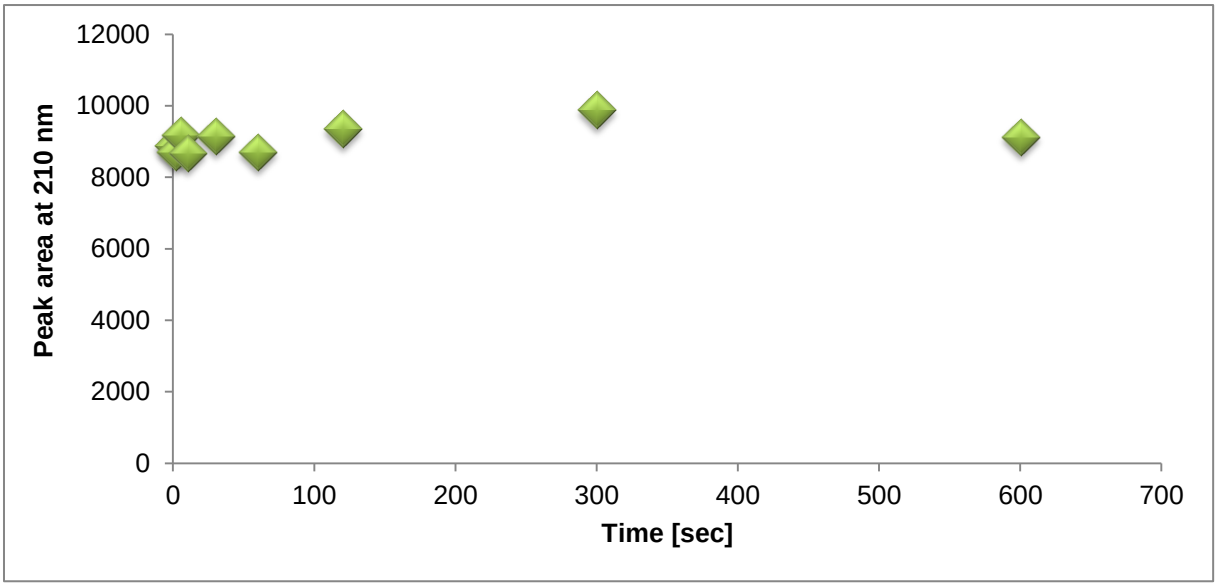


Figure 3.8.3 Rubusoside stability in phosphoric acid buffer pH=5



3.9 Steviosides in emulsions

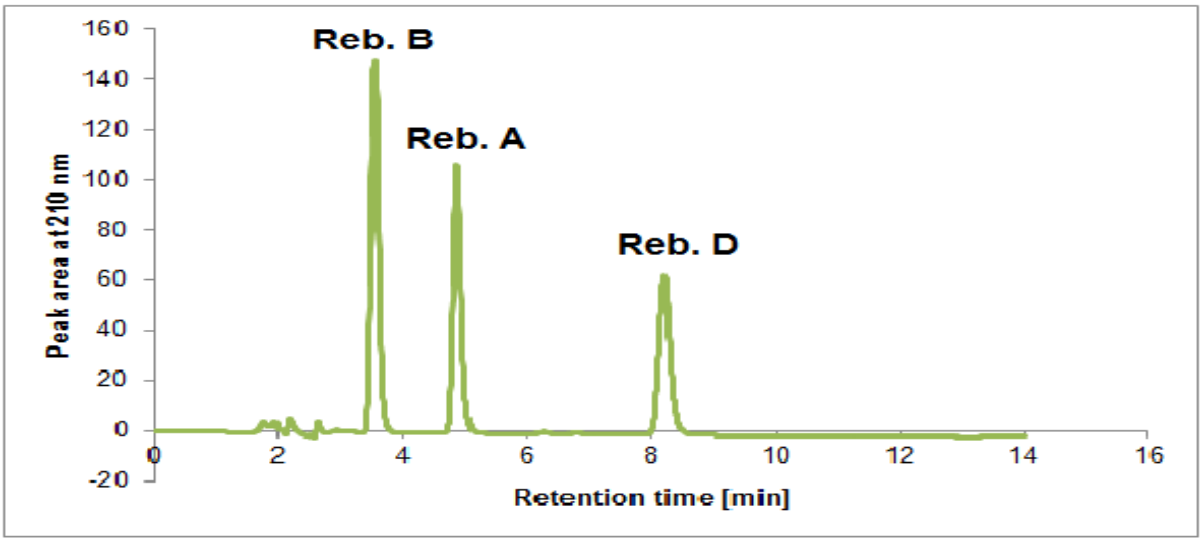


Figure 3.9.1 Chromatogram of steviosides A, B, and D, standards. Retention time was for reb. A 4.86 min, for reb. B 3.54 min and for reb. D 8.30 min under separation (75 % ACN, 25 % 10 mM $NaH_2PO_4 \times H_2O$ pH=3) at a flow rate 1 ml/min. Injection volume was 10 µl, pressure 85 bar, temperature 25 °C and stop time 14 min. Column APS-2 HYPERSIL (150 × 4.6 mm + 50 × 4.6 mm); concentration 0.5 mg/ml.

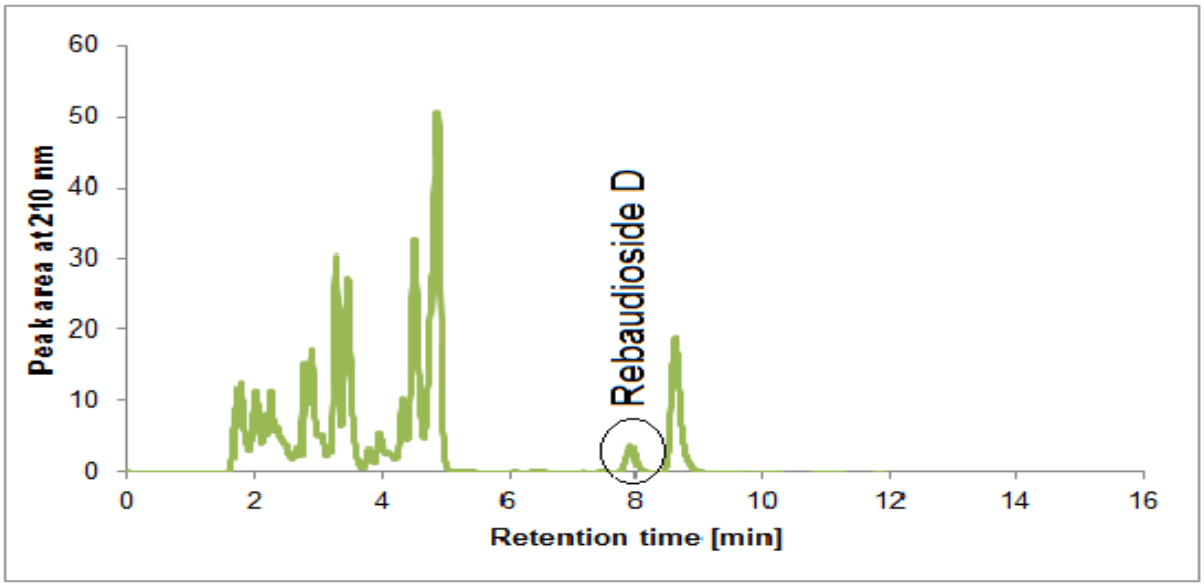


Figure 3.9.2 Chromatogram of steviosides in milk, aqueous phase. Retention time was 8.62 min under separation (75 % ACN, 25 % 10 mM $NaH_2PO_4 \times H_2O$ pH=3) at a flow rate 1 ml/min. Injection volume was 10 µl, pressure 85 bar, temperature 25 °C and stop time 14 min. Column APS-2 HYPERSIL (150 × 4.6 mm + 50 × 4.6 mm); concentration 0.5 mg/ml.

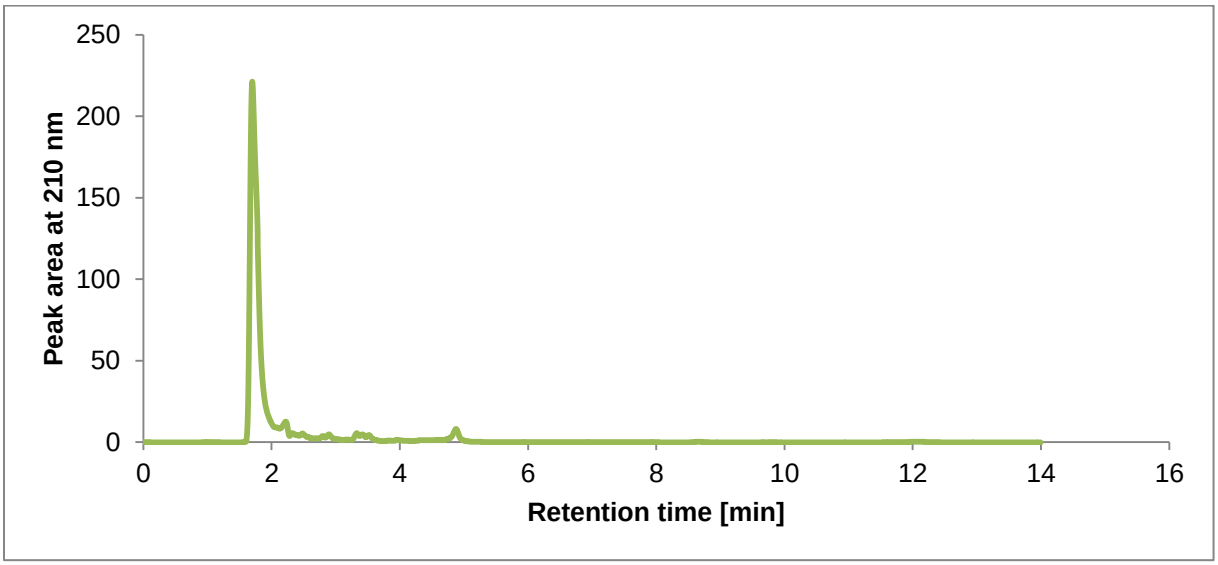


Figure 3.9.3 Chromatogram of steviosides in milk, fat phase. Under separation (75 % ACN, 25 % 10 mM $NaH_2PO_4 \times H_2O$ pH=3) at flow rate 1 ml/min and detection wavelength 210 nm. Injection volume was 10 µl, pressure 85 bar, temperature 25 °C and stop time 14 min. Column APS-2 HYPERSIL (150 × 4.6 mm + 50 × 4.6 mm); concentration 0.5 mg/ml.

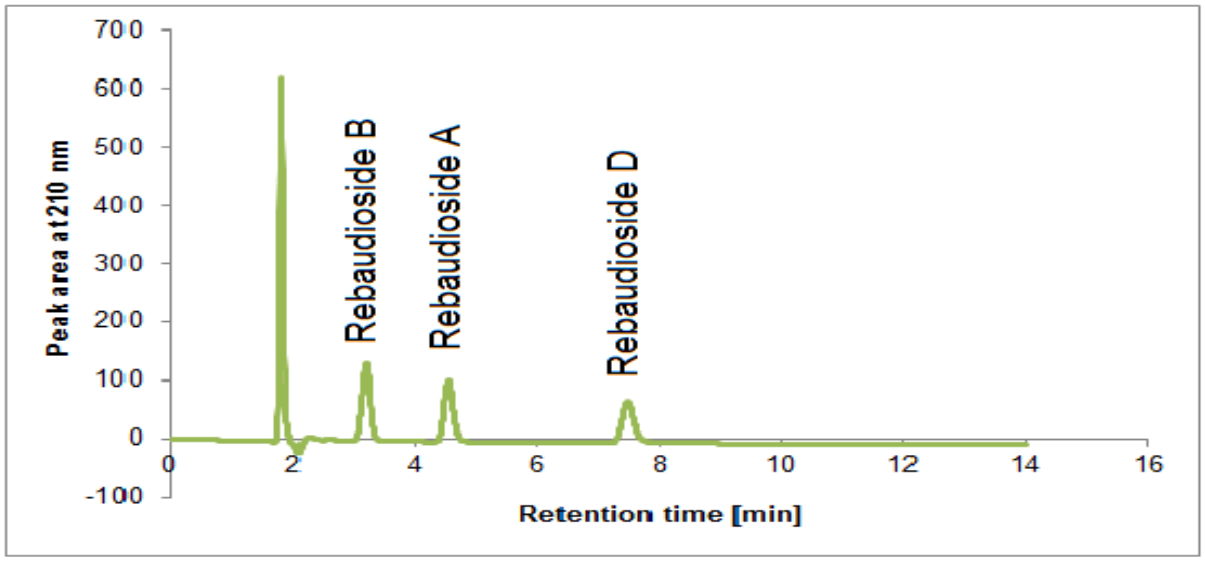


Figure 3.9.4 Chromatogram of steviosides in water-oil emulsion, aqueous phase. Retention time was 4.55 min for reb. A, 3.23 min for reb. B and 7.49 min for reb.D. Under separation (75 % ACN, 25 % 10 mM $NaH_2PO_4 \times H_2O$ pH=3) at a flow rate 1 ml/min. Injection volume was 10 µl, pressure 85 bar, temperature 25 °C and stop time 14 min. Column APS-2 HYPERSIL (150 × 4.6 mm + 50 × 4.6 mm); concentration 0.5 mg/ml.

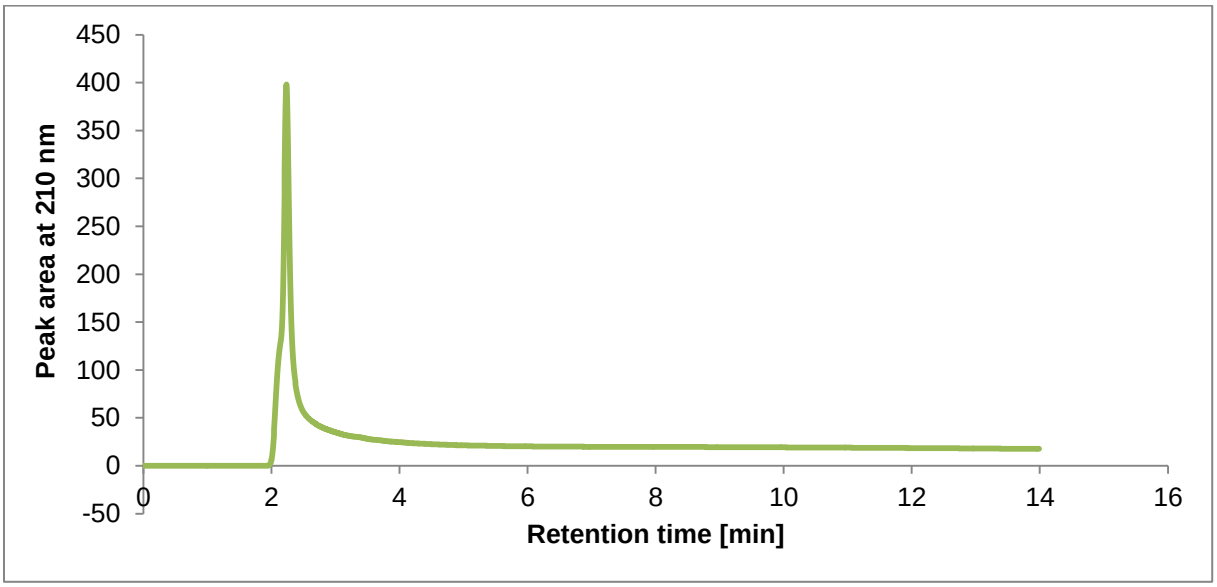


Figure 3.9.5 Chromatogram of water-oil emulsion without steviosides. Under separation (75 % ACN, 25 % 10 mM $NaH_2PO_4 \times H_2O$ pH=3) at a flow rate 1 ml/min and detection wavelength 210 nm. Injection volume was 10 µl, pressure 85 bar, temperature 25 °C and stop time 14 min. Column APS-2 HYPERSIL (150 × 4.6 mm + 50 × 4.6 mm).

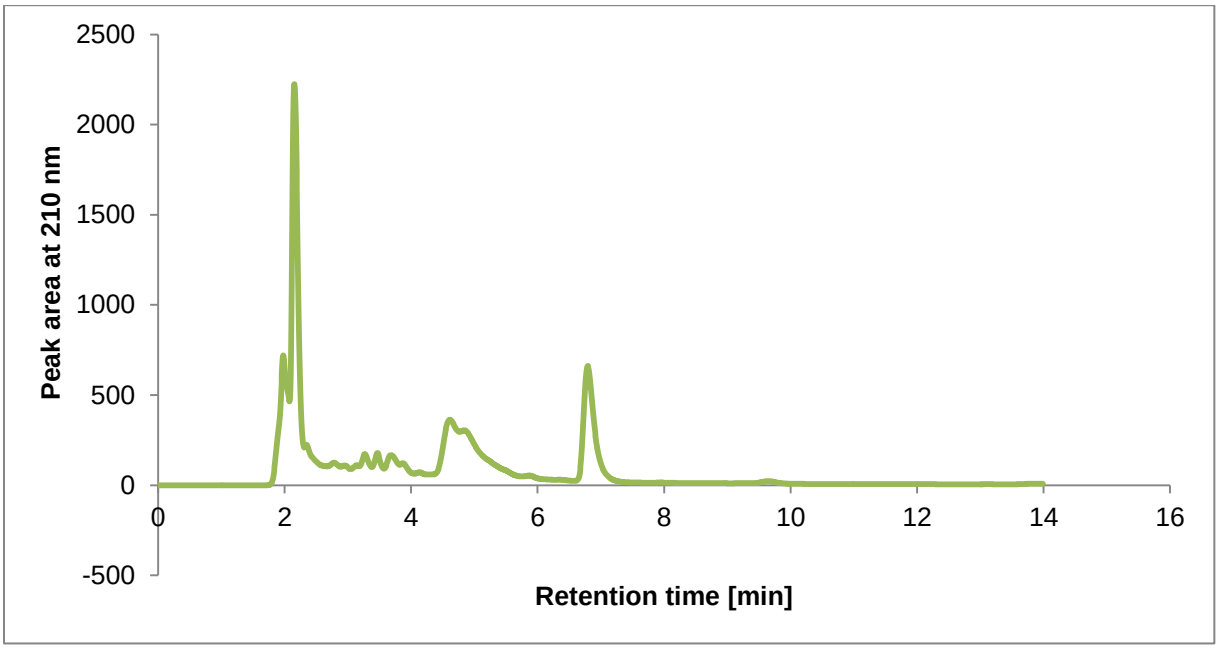


Figure 3.9.6 Chromatogram of espresso without steviosides. Under separation (75 % ACN, 25 % 10 mM $NaH_2PO_4 \times H_2O$ pH=3) at a flow rate 1 ml/min and detection wavelength 210 nm. Injection volume was 10 µl, pressure 85 bar, temperature 25 °C and stop time 14 min. Column APS-2 HYPERSIL (150 × 4.6 mm + 50 × 4.6 mm).

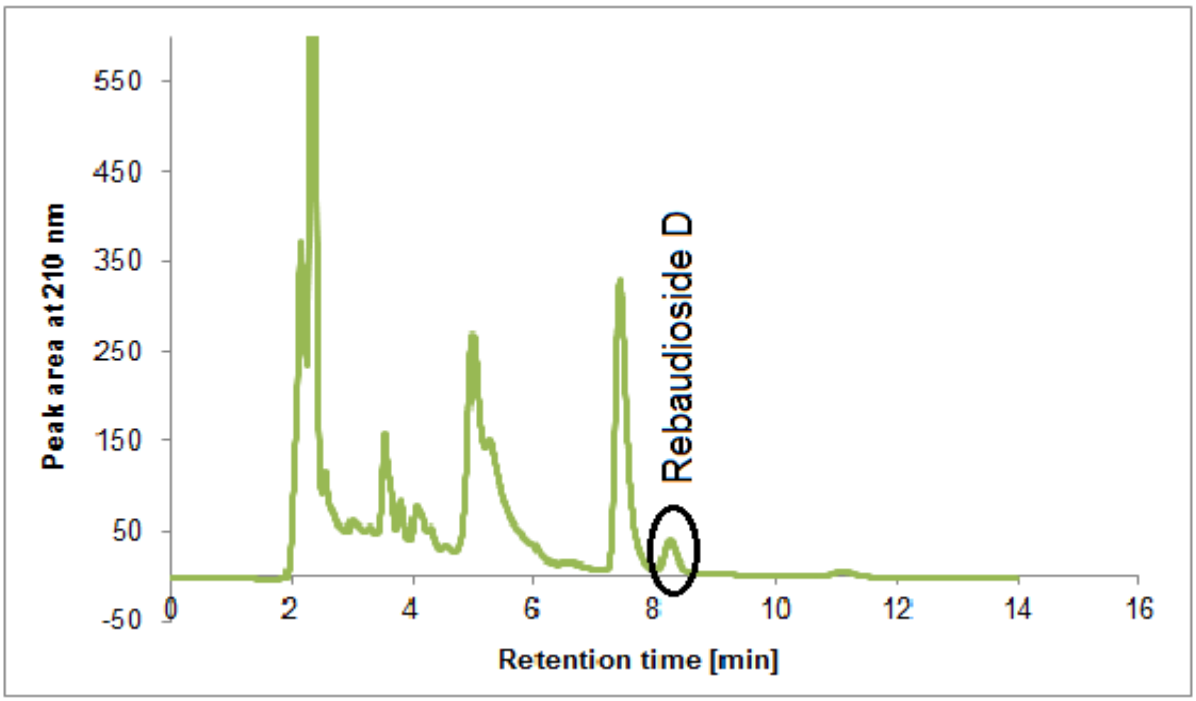


Figure 3.9.7 Chromatogram of steviosides in espresso. Retention time was for reb. D 8.25 min under separation (75 % ACN, 25 % 10 mM $NaH_2PO_4 \times H_2O$ pH=3) at a flow rate 1 ml/min. Injection volume was 10 µl, pressure 85 bar, temperature 25 °C and stop time 14 min. Column APS-2 HYPERSIL (150 × 4.6 mm + 50 × 4.6 mm); concentration 2 mg/ml.

4. Discussion

4.1 Stability in citric acid buffer (at room and refrigerator temperature)

Rebaudioside A and rebaudioside B showed good stability up to one week incubation in citric acid buffer pH values 2.6 - 6, at a temperature of 6 °C. After one week the stability decreased as shown in Figures 3.1.1 and 3.1.2.

Rebaudioside D showed good stability at pH 2.6 – pH 4 up to one week incubation at 6 °C, Figure 3.1.3.

Rubusoside stability is decreasing after the third day of incubation at pH 4, and rubusoside showed good stability at pH 5, pH 6 and pH 7 up to one week of incubation at 6 °C, Figure 3.1.4.

Stevioside also showed very good stability in citric acid buffer at room temperature (23 °C) stability. Rebaudioside A and rebaudioside D are stable up to one week at pH 2.6 – pH 5, shown in Figures 3.2.1 and 3.2.3.

Rebaudioside B shower good stability up to one week, whilst after one week the stability is decreasing, as shown in Figure 3.2.2.

Rubusoside showed good stability at pH 5 and pH 6 up to one week, whilst at pH 3 and pH 4 the stability decreasing after three days, as shown in Figure 3.2.4.

Rebaudioside A and rebaudioside D showed better stability then rebaudioside B and rubusoside. That was expected, because rebaudioside A and rebaudioside D have an additional glucose unit attached to the steviol core. They have more stable structure.

4.2 Stability in phosphoric acid buffer (at room and refrigerator temperature)

Rebaudioside A and rebaudioside B showed good stability in phosphoric acid buffer at 6 °C incubation up to one month. The stability decreased after one month, as shown in Figures 3.3.1 and 3.3.2.

Rebaudioside D showed good stability up to thirty days incubation in phosphoric acid buffer at 6 °C. At pH 3 decreasing of stability could be detected, after one month. At pH 4 and pH 5 slight decreasing of stability were detected, after one month, as shown in Figure 3.3.3.

Rubusoside showed good stability in phosphoric acid buffer at 6 °C incubation up to one week, no significant changes are occurred (Figure 3.3.4).

Incubation of rebaudioside A in phosphoric acid buffer at room temperature showed good stability up to one month. After one month decreasing of stability could be seen (Figure 3.4.1).

Incubation of rebaudioside B in phosphoric acid buffer at room temperature up to one month showed no significant change. After one month the stability is decreasing (Figure 3.4.2).

Incubation of rebaudioside D in phosphoric acid buffer at room temperature showed no change in stability (Figure 3.4.3).

Incubation of rubusoside in phosphoric acid buffer at room temperature showed decreasing of stability after one week (Figure 3.4.4).

Steviosides in phosphoric acid buffer are more stable then in citric acid buffer. Rebaudioside D, with five glucose units attached to the steviol core, showed no change in stability. It could be seen, that stability decreasing with lower pH, in both citric and phosphoric acid buffer.

4.3 Stability in citric acid buffer at elevated temperatures

Incubation of rebaudioside A in citric acid buffer pH 3 at 60 C°, 65 °C, 70 °C, 80 °C, 85 °C and 90 °, for 50 minutes showed very good stability, (Figure 3.5.1). At 95 °C decreasing of the stability after 20 minutes could be seen.

Rebaudioside A is remarkable stable in citric acid buffer pH 4 and pH 5, no change in stability after 50 minutes at 60 C°, 65 °C, 70 °C, 80 °C, 85 °C 90 °C and 95 °C (Figures 3.5.2 and 3.5.3).

Incubation of rebaudioside B in citric acid buffer pH 3, pH 4 and pH 5 at 60 C°, 65 °C, 70 °C, 80 °C, 85 °C, 90 °C and 95 °C (Figures 3.5.4, 3.5.5 and 3.5.6) showed no change in stability after 50 minutes.

Incubation of rebaudioside D in citric acid buffer pH 3 at 60 C°, 65 °C, 70 °C, 80 °C, 85 °C and 90 °, for 50 minutes showed very good stability, no change (Figure 3.5.7). At 95 °C decreasing of the stability after 20 minutes could be seen.

Rebaudioside D in citric acid buffer pH 4 and pH 5 (Figures 3.5.8 and 3.5.9) is stable over 50 minutes incubation at 60 C°, 65 °C, 70 °C, 80 °C, 85 °C, 90 °C and 95 °C.

4.4 Stability in phosphoric acid buffer at elevated temperatures

Rebaudioside A is stable in phosphoric acid buffer pH 4.2 and pH 5 for 50 minutes at 60 °C, 65 °C, 70 °C, 80 °C, 85 °C, 90 °C and 95 °C (Figures 3.6.2 and 3.6.3). At pH 3 after 30 minutes at 95 °C stability decreasing, as shown in Figure 3.6.1.

Rebaudioside B is stable in phosphoric acid buffer pH 4.2 and pH 5 i for 50 minutes at 60 °C, 65 °C, 70 °C, 80 °C, 85 °C, 90 °C and 95 °C (Figures 3.6.5 and 3.6.6). At pH 3 after 30 minutes at 95 °C stability decreasing, as shown in Figure 3.6.4.

Rebaudioside D is stable over pH range pH 3, pH 4.2 and pH 5 for 50 minutes at 60 °C, 65 °C, 70 °C, 80 °C, 85 °C, 90 °C and 95 °C, as shown in Figure 3.6.7; 3.6.8. and 3.6.9.

4.5 Rubusoside stability in citric and phosphoric acid buffer at 72 °C

Rubusoside in citric acid buffer is stable over wide pH range (pH 3 – 6), incubation for 10 minutes at 72 °C, as shown in Figures 3.7.2 – 3.7.5. Whilst, rubusoside in citric acid buffer pH 2.6 after 2 minutes stability is decreasing (Figure 3.7.1).

Rubusoside in phosphoric acid buffer is stable at pH 3, pH 4.2 and pH 5 after 10 minutes at 72 °C (Figures 3.8.1 – 3.8.3).

Rebaudioside D is stable under thermal treatment up to 95 °C, over a wide pH range (pH 3-6), in both citric acid buffer and phosphoric acid buffer.

Rebaudioside B and rebaudioside A undergo stability decreasing after 30 minutes at 95 °C. Rubusoside stability in citric acid buffer decreasing with lower pH (pH 2.6), while in phosphoric acid buffer rubusoside stays stable.

Steviosides shows good stability under normal condition of application. At elevated temperatures, steviosides showed better stability in citric acid buffer than in phosphoric acid buffer. The stability depends on temperature, storage time and pH value. It also depends on how much sugar molecules are attached to the steviol core.

Stability decreases with lower pH value (pH 2.6 or 3), higher temperature (> 90 °C), and longer time of storage (one week or one month). In this study, the best stability showed rebaudioside D in both buffers used.

4.6 Steviosides in emulsions

Steviosides behavior in emulsions was measured without pre-column and for that reason the retention times were shorter as follows for rebaudioside A 4.86 min, rebaudioside B 3.54 min, and for rebaudioside D 8.30 min.

As results show, steviosides tend to stay in the aqueous phase, in emulsions. In milk und espresso only rebaudioside D could be detected after 8.62 min, in the aqueous phase. In water-oil emulsion all three steviosides were detected in aqueous phase. The steviosides in the fat phase could not be detected.

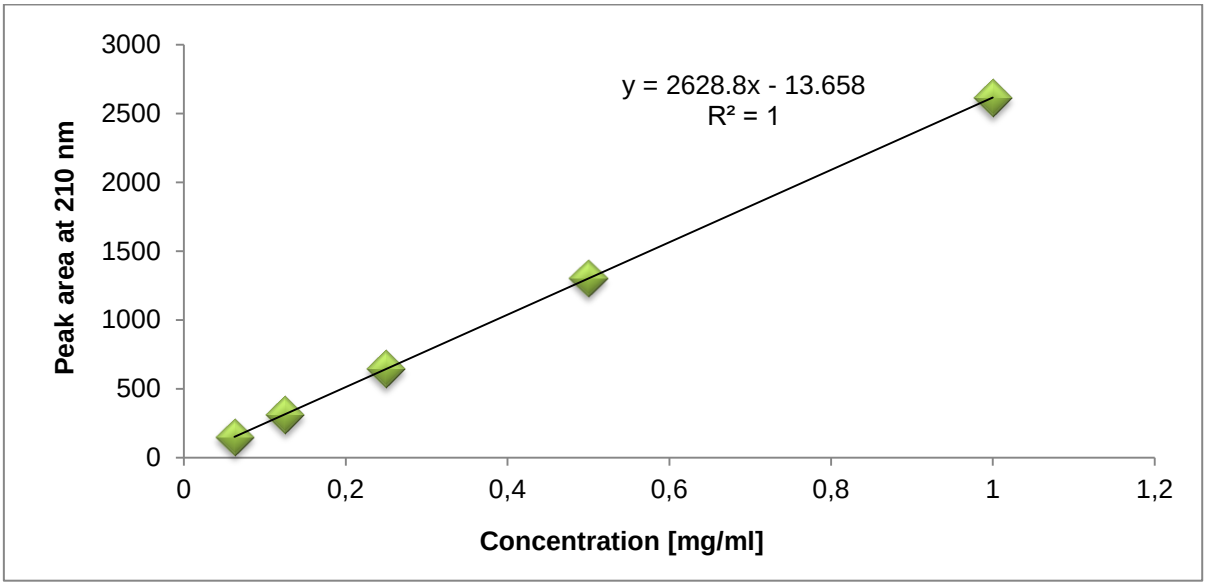
5. Appendix

Additional during my work the rubusoside content was analyzed in 17 samples. All samples are obtained from the Red Bull company. At first calibration curve was performed. The rubusoside standard was dissolved in 80 % ACN and 20 % H_2O, than solutions with different concentrations were prepared:

- 1 mg/ml
- 0.5 mg/ml
- 0.25 mg/ml
- 0.125 mg/ml
- 0.0625 mg/ml

Figure 5.1 ″Red Bull″ Probes

Table 5.1 Calibration curve for rubusoside content in ″Red Bull″ samples



For the calculation of the rubusoside concentration in ″Red Bull″ samples this equation was used: **y= 2628.8x - 13.658.**

Each probe were dilluted with mobile phase 1:1, and than analysed by HPLC. Table 5.2 lists conditions during measuring.

Table 5.2 HPLC conditions for rubusoside analysis in ″Red Bull″ samples

Parameter	Adjustment
Injection Volume	10 µl
Flow	1 ml/min
Temperature	25 °C
VWD Signal	210 nm
Stop time	8 min
Mobile phase	100 % (80 % ACN, 20 % H_2O)
Pressure	76 bar
Column	APS-2 HYPERSIL (150 × 4.6 mm + 50 × 4.6 mm)

Table 5.3 Rubusoside concentration in ″Red Bull″ samples

Sample	Area	X	Df (1:1) mg/ml	mg/100ml
GS	188.9	0.077053408	0.154106817	15.41
HTH	180.8	0.073972155	0.147944309	14.79
3.0.5	58.6	0.027487066	0.054974133	5.49
1.0.5	555.5	0.216508673	0.433017346	43.30
G				
1.4.3	633.9	0.246332167	0.492664334	49.26
1.4.4	610.9	0.237582928	0.475165855	47.51
1.4.5	586.4	0.228263086	0.456526172	45.65
3.4.3	110.1	0.047077754	0.094155508	9.41
3.4.4	57.7	0.027144705	0.05428941	5.42
3.4.5	63.3	0.029274954	0.058549909	5.85
1.8.3	807	0.312179702	0.624359404	62.43
1.8.4	803.4	0.310810256	0.621620511	62.16
1.8.5	825.8	0.319331254	0.638662508	**63.86**
3.8.3	112.8	0.048104839	0.096209677	9.62
3.8.4	110.3	0.047153834	0.094307669	9.43
3.8.5	109.7	0.046925593	0.093851187	9.38

Df=dilutions factor

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