9. Drudy, D. Characterization of a collection of Enterobacter sakazakii isolates from environmental and food sources [Text] / D. Drudy, M. O'Rourke, M. Murphy et al. // Int. J. Food Microbiol. – 2006. – Vol. 110, № 2. – P. 127–134.
10. Friedemann, M. Enterobacter sakazakii in food and beverages (other than infant formula and milk powder) [Text] / M. Friedemann // Int. J.Food Microbiol. – 2007. – Vol. 116. – P. 1–10.
11. Skladal, P. Detection of bacterial contamination in sterile UHT milk as L-lactate biosensor [Text] / P. Skladal, M. Mascini, C. Salvadori, G. Zannoni // Enzyme Microb. Technol. – 1993. – Vol. 15. – P. 508–512.
12. Muytjens, H. L. Enterobacter sakazakii meningitis in neonates: causative role of formula [Text] / H. L. Muytjens, L. A. Kollee / Pediatric Infectious Disease. – 1990. – Vol. 9. – P. 372–385.
13. Salmon, S. A. Minimum inhibitory concentrations for selected antimicrobial agents against organisms isolated from the mammary glands of dairy heifers in New Zealand and Denmark [Text] / S. A. Salmon, J. L. Watts, F. M. Aarestrup et al. // J. Dairy Sci. – 1998. – Vol. 81. – P. 570–578.
14. Berhilevych, A. Methods of isolation and counting of bacteria Enterobacter (Cronobacter) sakazakii [Text] / A. Berhilevych, V. Kasyanchuk, T. Garkavenko, NA Mezhynska / / Approved scientific methods Council of State Committee for Veterinary Medicine of Ukraine, 2010 - 38 p.
15. ISO/TS 22964:2006, Milk and milk products - Detection of Enterobacter sakazakii.
16. Patent of Ukraine for useful model № 86516. Method of fixing microorganisms of the fixator Trumpsa 4F: 1G to study their ultra-structure under scanning electron mikrosokpom [Text] / Berhilevych OM, Kasyanchuk VV Berhilevych OO, Mohutova VF / / applicant Sumy NAU. - Zavl. 26/11/2012; publ. 1. 01.2014 Bull. Number 1. - 5 seconds.
17. Patent of Ukraine for useful model № 86516. Method of fixing microorganisms lock Trumpsa 4F: 1G to study their ultrastructure under scanning electron mikrosokpom [Text] / Berhilevych O., Kasyanchuk V., Berhilevych O., Mohutova V. // applicant Sumy NAU. - Zavl. 26/11/2012; publ. 1. 01.2014 Bull. Number 1. - 5 seconds.

УДК 633.3:658.562

STUDY OF THERMODYNAMICS OF COMPLEX FORMATION OF FLAVONOIDS OF STEVIA (STEVIA REBAUDIANA BERTONI) LEAVES

I. Kuznetsova
Candidate of Engineering Sciences,
Senior researcher
Sector of researches and control of stevia quality indexes
Institute for Bioenergy Crops and Sugar Beet of NAAS
Klinichna str., 25, Kiev, Ukraine, 03141
E-mail: ingaV@ukr.net

Вивчено механізм утворення комплексу флаваноїдів з іонами алюмінію. На основі від'ємних низьких значень енергії Гіббса встановлено, що комплексоутворення є природним явищем. Вперше визначено термодинамічні властивості флаваноїдів листків стевії (Stevia rebaudiana Bertoni) сушеної та розроблено номограму залежності вмісту флаваноїдів від ймовірності ступеня комплексоутворення. У результаті проведених досліджень визначено антиоксиданту здатність флаваноїдів, що містяться в листках стевії

Ключові слова: листки стевії, флаваноїди, ступінь комплексоутворення, енергія Гіббса, константа стійкості

Изучен механизм образования комплекса флаваноидов с ионами алюминия. На основе отрицательных низких значений энергии Гиббса установлено,что комплексообразование является природным явлением. Впервые определены термодинамические свойства флаваноидов листьев стевии (Stevia rebaudiana Bertoni) сушенных и разработана номограмма зависимости содержания флаваноидов от возможности комплексообразования. В результате проведённых иследований определена антиоксидантная способность флаваноидов, которые содержатся в листьях стевии

Ключевые слова: листья стевии, флаваноиды, степень комплексообразования, энергия Гиббса, константа стойкости

1. Introduction

Flavonoids are natural herbal compounds, which show biological, antioxidant, protective, antiradical, antibacterial, antiviral, antiinflammatory and vasodilating abilities. A separate group is represented by flavolignans, which are the condensed products of dihydroquercetin with lignin, show biological activity, the basic of which is hepatoprotective. One of such abilities is forming complexes with metals (e. g. iron and copper) that reduces the content of metals in a human body and reduces the generation rate of reactive oxygen species, thus protecting biologically active molecules of the body from an oxidizing process [1, 2].

The current ecological situation requires eliminating metal ions, which have a toxic effect from the organism of man and others. In particular, such metal as aluminium, contained in drinking water in excessive amounts as a result of «acid rains» or using the aluminium-based flocculant for water treatment. Aluminium has a toxic effect, depending on the form, in which it is contained in water - inorganic monomeric aluminium, which includes hydrated ions or aquacomplexes $[Al(H_2O)_6]^{3+}$ and hydrocomplexes $Al(OH)^{2+}$ and $Al(OH)_2$. In addition, aluminium sulfate $Al_2(SO_4)_3$ is used mainly during drinking water treatment to remove jellies and organic matters that leads to an increase in the inorganic monomeric aluminium content. Toxicity of aluminium appears in the human body mostly in the form disturbance in metabolism, especially mineral, nervous system in the cell reproduction and growth part [3]. Taking into account the toxic effect of aluminium on the human body, the European Union and the World Health Organization have set the limiting content of aluminium in drinking water at the level of 200 mcg/l, and the recommended concentration is 50 mcg/l [4].

Ions of harmful metals can be removed from the human body by natural compounds, such as flavonoids. Based on previous studies, it was found that dried stevia (*Stevia rebaudiana Bertoni*) leaves is a rich source of flavonoids [5] and can be used for removing aluminium ions from the human body.

2. Analysis of literary data and problem statement

The basis of chemical structure of flavonoids is a flavonoid kernel, which consists of 15 carbon atoms, which form three rings (C6-C3-C6): A, B and C (Fig. 1). The classes of flavonoids are divided according to the C ring oxidation level. In these classes, separate flavonoids differ by the presence of hydroxy- or oxysubstitutes in the A and B rings, which determine the antioxidant property [6]. The B ring hydroxy groups, the amount of which affect the complex formation degree and antioxidant effect, are involved in the formation of the complex [7].

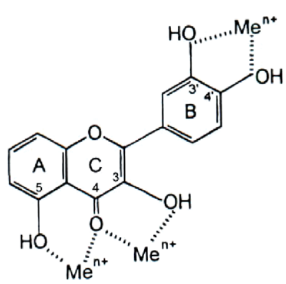

Fig. 1. General structure of the formed complex of flavonoid with metal ions

There are two basic mechanisms of antioxidant action of flavonoids: acceptance of free radicals and chelation of metal ions with the formation of complexes [8]. Flavonoids are able to react with organic and alkoxyl radicals of different compounds, radicals of aromatic aminoacids, anion ascorbates, etc. Most flavonoids can form complexes with metal ions. Thus, the properties of flavonoids in the formed complex change, showing high reactivity with respect to the anion radical [9]. The mechanisms of forming such complexes between flavonoids (quercetin, morin, taxifolin, silybin A) and ions of iron and copper [1] were studied. Today, much emphasis is placed on studying the molecular formation of complexes of vegetable triterpene glycosides with different bioactive compounds, which promote reducing therapeutic doses, improving solubility, expanding biological activity of medicinal substances [7, 10]. In particular, it is acknowledged that stevia is a plant, rich in bioactive compounds and shows functional ability due to flavonoids [11, 12].

The purpose of the work is to study the mechanism of forming the complex between flavonoids of stevia leaves and aluminium ions and the stability level of the formed complex.

However, today the antioxidant action of flavonoids of stevia leaves is not studied and description of thermodynamics, namely in relation to the possibility of the natural flow of the complex formation process is not given. In addition, much attention in world studies is paid to the interaction of flavonoids with the ions of iron and copper. However, taking into account imperfection of drinking water treatment, which causes the greater aluminium content, it is important to study the problem of removing aluminium ions from the human body.

3. Methods and techniques of research

Stevia, grown in the «Agro-company «Veselinovka» (Kyiv region), which, after reaching the buddling phase, was cut and dried at a temperature 60 °C by the convection method was used in researches. The obtained dry leaves were analyzed for the degree of antioxidant action.

For the analysis, aqueous extract from the dried stevia (*Stevia rebaudiana Bertoni*) leaves was obtained and, using the spectrophotometric method [13], the content of flavonoids, which was 561 mg/l, was determined. A solution of 5 % aluminium chloride in ethanol was added in different ratios (1:0, 3.1:15) to 1 ml of the extract and the volume was increased up to 10 ml by ethyl alcohol (60 %). The solution was left in a dark place for 30 min, then filtered and the optical density was determined on the SP-46 at the wavelength of 410 nm.

4. Presentation of basic research results

Dried stevia (*Stevia rebaudiana Bertoni*) leaves is the multicomponent biosystem, containing compounds, which in total determine their biological value.

According to the law of mass action, the biosystem, which contains flavonoids (mM) forms the complex (M_mR_n) with aluminium ions (R_n), which are in excessive amount (1):

$$mM + nR \rightleftarrows M_m R_n \, . \quad (1)$$

Determining stoichiometric ratios of ligands was carried out by the Ostromyslenskiy-Job method (continuous measurement method) [14] based on determining the ratio of isomolar concentrations of reactants, corresponding to the maximum output of the formed complex.

The catechol part of the B-ring of flavonoids, 3-hydroxy- and 4-oxy groups of heterocyclic C-ring, 4-oxy- and

5-hydroxy groups of heterocyclic C- and A-rings enter into the complex formation reaction with metals. The B-ring groups are the most involved in the formation of complexes. Flavonoids absorb UV light in the visible spectral regions: band I – 320-420 nm, band II – 280-285 nm. The band I is peculiar to the B-ring and the band II – to the A-ring. Absorption of light in these ranges takes place due to π-π* by transitions of electrons [1].

The obtained curve (Fig. 2) of dependence of the complex output on the amount of flavonoids of the extract from stevia leaves is characterized by an extreme point (0,65) and indicates the maximum possible concentration of the ligand complex with the aluminium ion. The position of extremum is connected with stoichiometric coefficients [15].

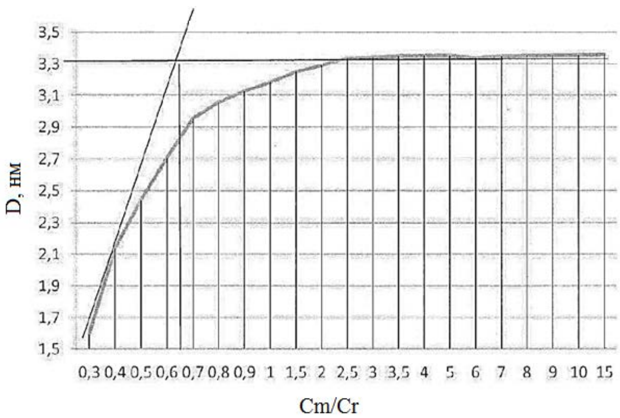

Fig. 2. Dependence of optical density on the ratio of components of izomolar series

Taking into account that the extract, obtained from the dried stevia (*Stevia rebaudiana Bertoni*) leaves contains 561 mg/l of flavonoids, 364,7 mg/l of flavonoids is spent on forming the complex with aluminium ions. Increasing the amount of extracted aluminium ions is possible at increasing the introduced amount of flavonoids.

Reducing the content of aluminium ions due to forming complexes with flavonoids of stevia leaves decreases the generation rate of reactive oxygen species, and, as a result, increases protection of biologically active molecules of the body against the oxidizing process [12].

According to the experimental data on the content of flavonoids in dried leaves of stevia (*Stevia rebaudiana Bertoni*), grown in different agrotechnic conditions of Ukraine (Table 1), using the Klotz method [14], the complex formation degree and the stability constant of flavonoids in solution (Table 2) were calculated. The Klotz method is used in calculations for moderately strong solutions, which also include the extract, obtained from the dried stevia (*Stevia rebaudiana Bertoni*) leaves.

Table 1

Content of flavonoids in dried leaves of stevia (Stevia rebaudiana Bertoni)

Growing stevia	Content of flavonoids, mg/l
Kyiv region	657±0,1
Vinnytsya region	643±0,1
Zhytomyr region	638±0,1
AR Crimea	620±0,1

Determining the complex formation degree and the stability constant includes the component system of the extract and probable efficiency of cleaning. The complex formation degree (χ) determines the complex-stability constant (β):

$$\beta = \frac{M_m R_n}{mM + nR}. \quad (2)$$

The concentration stability constant (β) changes at changing the activity coefficients of acting particles that, respectively, affects the complex formation degree and changes at changing the total ionic strength of the solution. In addition, the complex formation degree will depend on the equilibrium concentrations of flavonoids and aluminium ions.

Using the value of the instability constant (C), the stability constant of complexes and Gibbs free energy (at T=293 K) was calculated. The obtained thermodynamic feature of flavonoids of stevia, grown in different climatic conditions (Table 2) shows that forming the complex with aluminium ions is a natural phenomenon, which occurs without applying any efforts (heating, etc.) that is confirmed by the low negative value of the Gibbs energy.

Table 2

Thermodynamic parameters of forming the complex of flavonoids with aluminium ions in aqueous solutions at a temperature 20 °C

Region	Stability constant (β), l/mol	Instability constant (K), mol/l	Complex formation degree (χ)	lgβ	ΔG293, kJ/mol
Kyiv	200	5×10^{-3}	0,6	2,3	-12,89
Vinnytsya	262,5	$3{,}8\times10^{-3}$	0,15	2,42	-13,55
Zhytomyr	250	4×10^{-3}	0,41	2,4	-13,45
AR Crimea	290	$3{,}5\times10^{-3}$	0,41	2,46	-13,78
Total	250,63	$4{,}08\times10^{-3}$	0,4	2,4	-13,42

One of flavonoids - quercetin forms complexes in acidic medium with 1:1 stoichiometry, in which 3-hydroxy- and 4-oxy groups of the C-ring are involved, in alkaline medium – due to the participation of the catechol group of complex with high stoichiometry – Me:Ligand [13]. The change in the structure of flavonoids affects the complex formation degree. Analysis of the stability of the formed complex shows that maximum stability is provided by the presence of double bonds in the C-ring. Thus, the strength of the formed complexes with the participation of stevia leaves flavonoids is achieved due to rutin and quercetin.

According to the values of the complex formation degree of flavonoid complex, graphic dependence of flavonoids (ROS) of the leaves of stevia, grown in different agroclimatic zones of Ukraine on the complex formation (Fig. 3) was built.

The obtained dependence can be used when determining the content of flavonoids in stevia leaves for calculating their complex formation degree. Using the obtained nomogram, by the value of the content of flavonoids in the extract from stevia leaves, the degree of forming the complexes with aluminium ions was determined that simplifies calculations and allows to define the aluminium ions removal degree in a short period.

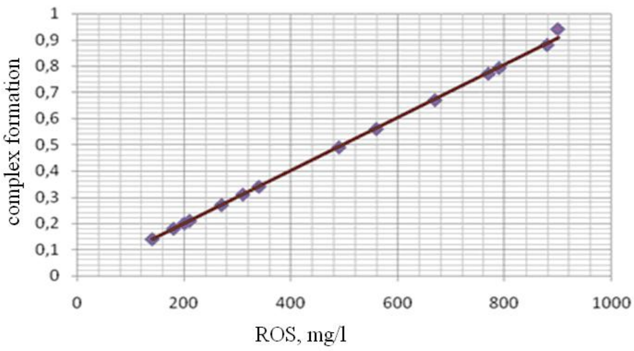

Fig. 3. Change of the complex formation degree depending on the content of flavonoids in dried leaves of stevia, grown in different agroclimatic zones of Ukraine

5. Conclusions

It is found that stevia leaves is the rich source of flavonoids (620–650 mg/l), which take active part in the comlex-formation and have a high antioxidant effect. Thermodynamic parameters of flavonoids of dried leaves of stevia (*Stevia rebaudiana Bertoni*) with aluminium ions were first calculated. The mechanism of forming the complex of flavonoids with aluminium ions: the influence of the content of flavonoids on the aluminium ions removal degree is studied. It was determined that 65 % of flavonoids of stevia leaves enter into the complex formation reaction. The Gibbs energy of stevia flavonoids is 12,8–13,8 that indicates the natural flow of the complex formation process. Based on the data on the content of flavonoids in stevia leaves, the nomogram of the degree of forming the complex of flavonoids of stevia leaves with aluminium ions depending on the agroclimatic zone of its growing was developed. Using the nomogram will simplify the complex formation calculation.

References

1. Schekatikhina, A. S. Spektrofotometricheskaya description of kompleksov kvercetina, morina, taksifolina I silibina s ionami honeys (II) [Text] / A. S. Schekatikhina, V. P. Kurchenko // Trudy BGU. – 2011.– Vol. 6, Part 1. – P. 76–85.
2. Piett, P.-G. Flavonoids as antioxidants [Text] / P.-G. Piett // J. Nat. Prod. – 2000. – № 63. – P. 1035–1042.
3. Zhezherya, V. A. Content and forms of finding of aluminium is in water of the rivers of pool of Prip'yati [Text] / V. A. Zhezherya, P. M. Linnik // Sciences. labours of UKRNDGMI. – 2010. – Issue 259. – P. 171–187.
4. Linnik, P. N. Nekotorykh osobennostyakh kompleksoobrazovaniya of Al(III) of s gumusovymi veschestvami [Text] / P. N. Linnik, V. A. Zhezherya, R. N. Linnik // Metody of I ob'ekty khimicheskogo analiza. – 2009. – Vol. 4, № 1. – P. 73–84.
5. Kuznetcova, I. V. Role of phenols and flavanoidiv in the estimation of quality of stevia (Stevia rebaudiana Bertoni) [Text] / I. V. Kuznecova // Newest technologies of growing of agricultural cultures: Zb. sciences. pr. / for the editor of akad. NAAN Roika M.V. – 2013. – Issue 14. – P. 146–150.
6. Burda, S. Antioxidant and antiradical activities of flavanoids [Text] / S. Burda, W. Oleszek // J. Agric. Food Chem. – 2001. – № 49. – P. 2774–2779.
7. Pedrielli, P. Antioxidant mechanism of flavonoids. Solvent effect on rate constant for chainbreaking reaction of quercetion and epicatechin in autoxidation of mathyl linoleate [Text] / P. Pedrielli, G. F. Pedulli, L. H. Skibsted // J. Agric. Food Chem. – 2001. – № 49. – P. 3034–3040.
8. Pecal, A. Interaction of quercetin with copper ions: complexation, oxidation and reactivity towars radicals [Text] / A. Pecal, M. Biesaga, K. Pyrzynska // Biometals. – 2010. – № 330. – P. 1173–1178.
9. Chernyakovskiy, E. M. Role of flavanoidov biologicheskikh reakciyakh by a transfer electrons [Electronic resurs] / E. M. Chernyakovskiy, V. P. Kurchenko, V. A. Kostyuk. – Available at: http://www.bio.bsu.by/proceedings/articles/2009-4-1-9-26.pdf.
10. Yakovishin, L. A. Issledovanie of thermodynamics of molecular kompleksoobrazovaniya of saponinov ivy by a method spectrophotometers [Text] / L. A. Yakovishin, M. A. Rubinson, V. I. Grishkovec // Uchenye of message Tavricheskogo of national universiteta im. V. I. Vernadskogo. Seriya «Fiziko-matematicheskie sciences». – 2012. – Vol. 25(64), № 1. – P. 212–216.
11. Tolstikova, T. G. The complexes of drugs with carbohydrate-containing plant metabolites as pharmacologically promising agents [Text] / T. G. Tolstikova, M. V. Khvostov, A. O. Bryzgalov // Mini Rev. Med. Chem. – 2009. – Vol. 9, № 11. – P. 1317–1328.
12. Savita, S. M. Stevia rebaudiana-A functional component for food industry [Text] / S. M. Savita, K. Sheela, S. Sananda // J. Hum. Ecol. – 2004. – № 15. – P. 261–264.
13. Patent of Ukraine on an useful model, MPK9 S07 N 15/00, S07 N 17/00, G01 N a 21/00 Method of determination content of matters flavanoids complex [Text] / Roik M. V., Kuznecova I. V, Rudakova T. V. // declarant patentovolodar Kyiv. In-t of biopower cultures and sugar beets of NAAN. – № u201211791 zayavl. in 12.10.12.
14. Bulatov, M. I. Prakticheskoe of rukovodstvo for to the fotometricheskim methods of analiza [Text] / M. I. Bulatov, I. P. Kalinkin; 5th izd. – L.: Khimiya, 1986. – 432 p.
15. Panhwar, Q. K. Synthesis, characterization, sprctroscopic and antioxidant studies of Cu(II)-morin complex [Text] / Q. K. Panhwar, Sh. Mamon, M. I. Bhanger // Journal of molecular structure. – 2010. – № 967. – P. 47–53.