Scientific Bulletin. Series F. Biotechnologies, Vol. XXIX, No. 1, 2025
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

INFLUENCE OF NATURAL AND SYNTHETIC SWEETENERS ON FLAVOPROTEINS FOUND IN BLACK TEA

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Abstract

Black tea is a very popular drink and is consumed worldwide for its flavor and health benefits. Due to its powerful antioxidant compounds, drinking black tea has health benefits for the consumer. Flavoproteins are one of the most widely studied families of enzymes - proteins that contain a nucleic acid derivative of riboflavin. Flavoproteins have either FMN (flavin mononucleotide) or FAD (flavin adenine dinucleotide) as a protein group or cofactor. The majority of flavoproteins, carry out redox reactions. This work paper aims to study the sweetener-induced changes in flavoproteins. The results showed very significant changes induced by honey (V_3) and saccharin (V_4) on the concentrations of the active forms of flavoprotein in black tea.

Key words*: antioxidant, black tea, FAD, flavoproteins, FMN.*

INTRODUCTION

Tea, made from the leaves of the *Camellia sinensis* plant, is one of the most popular and widely consumed beverages in the world. It has many health benefits, with a specific taste and flavor. Black tea has a very valuable chemical composition, and it has several chemical compounds with a powerful antioxidant effect (Ye et al., 2021).

The notion of black tea as 'living food' is based on its chemical composition, which is extremely valuable, and its biodynamic properties, which allow concentration changes in nanoseconds (Savescu, 2017b).

Consumer demand for dietary supplements is growing, especially those with high antioxidant potential due to their ability to combat free radicals (Belitz et al., 2009; Savescu, 2017c).

Riboflavin is an alloxazine derivative consisting of an isoalloxazine nucleus and a ribitol side chain.

Riboflavin is known as the 'growth vitamin' and plays an important role in the body's oxygen reactions. It works with other substances called NAD-dependent oxidoreductases to help the body grow and absorb vitamin B3 (Savescu, 2017b). Vitamin B2 is found in the structure of its coenzyme forms, FMN and FAD (Savescu, 2020 b). It is converted to a monophosphoric ester (FMN) in all the body's cells when acted upon by a flavo-kinase. FAD-dependent oxidoreductases are another type of enzyme that utilizes vitamin B2 (riboflavin or 7,8-dimethyl-10-dimethyl-10-ribiethyl-isoaloxazine) (Savescu, 2017a, 2017b). These enzymes are found in the aerobic dehydrogenase group. Flavin enzymes (FMN, FAD) are involved in reactions involving the transfer of electrons and protons, which are mediated by the isoalloxazine nucleus (Savescu, 2021).

Flavin enzymes are different from NAD^+ and $NADP^+$ because they can either accept one electron or a pair of electrons. Flavine enzymes have standard redox potentials Eo between +0.19V (stronger oxidizers than NAD^+) and -0.49V (stronger reducing agent than NADH). (Savescu, 2017b).

This shows that their redox properties can vary a lot depending on the environment and the substrate. Some flavin enzymes contain a metal (molybdenum or iron) in their molecule. These enzymes can stabilize the semiquinone form by pairing the lone electron with the unpaired electrons existing in the metal ions. The metal can also help the flavin enzymes to transport electrons (Savescu, 2021).

Succinate dehydrogenase is an enzyme that works by removing two hydrogen atoms from succinic acid and transferring them to the FAD group (Skoog, 2007; Savescu, 2016a).

Many of these flavin enzymes also contain a metal that is essential for the function of metal-flavoprotein enzymes. FAD is also involved in the process of converting fatty acids to water, which is the first step in the process of oxidation (Savescu et al., 2019).
As demonstrated by Nobel Laureate Szent-Györgyi, flavoproteins function as electron carriers between dehydrogenase and iron-containing cytochrome enzymes. The electron transport chain segments were reconstituted in vitro using purified components (Isenberg et al., 1958).
Vitamin B3 (niacin, vitamin PP) is represented by two forms: nicotinic acid (niacin) and nicotinic acid amide (niacin amide). This vitamin functions in oxidoreduction reactions within the body, in the form of two coenzymes: NAD^+ (nicotinamide adenine dinucleotide) and $NADP^+$ (nicotinamide adenine dinucleotide phosphate). The process of electron collection from various substrates by NAD^+-dependent dehydrogenase enzymes is known as the '$NADH+H^+$' process. These electrons are subsequently transferred along the respiratory chain via flavoproteins and NADH-dehydrogenases (Savescu, 2017c; Tita et al., 2021).
Two discrete categories of flavoproteins possess dehydrogenase activity. L-amino acid oxidase is localized in the endoplasmic reticulum, whereas D-amino acid oxidase is found in the microsomes of liver cells (Savescu, 2016b). Some parts of the oxidases ($FADH_2$ and $FMNH_2$) react directly with molecular oxygen, generating H_2O_2. After this, catalase catalyzes the decomposition of H_2O_2 into water and oxygen. This process takes place in the peroxisomes of liver cells (Savescu et al., 2019).
Flavin mononucleotide (FMN) is a prosthetic group of flavoprotein. It is similar in structure to flavin adenine dinucleotide (FAD), but lacks adenine as a nucleotide (Savescu, 2017b). FMN (like FAD) can accept two electrons and two protons to yield $FMNH_2$. When bound to the active site of some enzymes, FMN can accept electrons, converting to a semiquinone radical (Savescu et al., 2020a).
It is estimated that approximately 90% of flavoproteins are classified as oxidoreductases. These enzymes are involved in a wide range of cellular processes. Flavins are a type of molecule that can change color and are found in many different proteins (Figure 1). Flavins come from vitamin B2 and are found in proteins such as flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN). (Toogood et al., 2020; Zhuang et al., 2022).

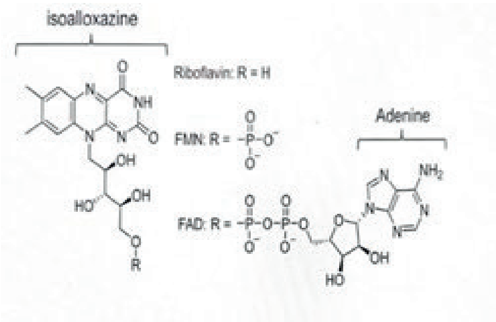

Figure 1. Molecular structure of flavins (Zhuang et al., 2022)

MATERIALS AND METHODS

The effect of various natural and synthetic sweeteners on the chemical composition of black tea has been studied in the laboratory.


Figure 2. Experimental variants

In order to obtain the control variant (V_0) of unsweetened black tea, 10 grams of black tea leaves were added to 1 liter of water in a suitable container. The tea was then subjected to a heating, cooling, and filtration process to yield the experimental variant from which the other sweetened black tea variants were derived (Figure 2).
The production of ten sweetened tea variants was undertaken from this experimental variant. The following variants were obtained, and are highlighted below:
V_0: a variety of unsweetened black tea;

V_1: a variety of black tea sweetened with white sugar;
V_2: a variety of black tea sweetened with brown sugar;
V_3: a variety of black tea sweetened with honey;
V_4: a variety of black tea sweetened with saccharin;
V_5: a variety of black tea sweetened with Sucrazit;
V_6: a variety of black tea sweetened with Diamond;
V_7: a variety of black tea sweetened with fructose;
V_8: a variety of black tea sweetened with xylitol;
V_9: a variety of black tea sweetened with sorbitol;
V_{10}: a variety of black tea sweetened with stevia.

The experimental version Sucrazit (V_5) is the trade name of a synthetic sweetener containing 25% saccharin, citric acid, whose acidity has been buffered with sodium bicarbonate.

The experimental variant (V_6) Diamond is the trade name for a synthetic sweetener containing a combination of sodium cyclamate and sodium saccharin.

For this research, Certified Reference Materials (Pure Analysis substances) were used, with the help of which calibration scales with different concentrations were made, which helped to find the wavelength at which the maximum molecular absorption spectra for flavoproteins and riboflavin are recorded.

As demonstrated in our previous study, a dilution of 2% was employed to enable the transmission of the incident light beam through the black tea solution. This dilution was obtained by passing the black tea through filter paper and its subsequent transfer to the spectrophotometer cuvettes for analysis (Butoi et al., 2023).

A UV-VIS T92 Plus spectrophotometer from PG Instruments (UK) was used to obtain the molecular absorption spectra of the experimental variants. The spectrophotometer has been configured to operate with a wavelength bandwidth of one centimeter. This configuration permits the measurement of nanometers of molecular absorption in the ultraviolet (UV) range of 190-400 nanometers and in the visual (VIS) range of 400-700 nanometers (Butoi et al., 2023).

Absorption measurements were conducted utilizing UV cuvettes composed of quartz and exhibiting a parallelepiped configuration, with a square side measuring 1 cm. Before analysis, the raw material was tested using A.A.S. (Atomic Absorption Spectroscopy) to check for any contamination such as residues or heavy metals (L'vov, 1990). The effect of sweeteners on the base chemical composition was investigated by utilizing UV-VIS optical spectrometry and mathematical statistics (Savescu, 2017b; Butoi et al., 2023).

The initial phase of sample preparation for atomic absorption spectrometry entailed mineralization, which involved microwave digestion (Savescu, 2017c).

Savescu showed in his paper that the process of microwave digestion constitutes a technique that has been utilized to prepare samples originating from a variety of sources (e.g., plants, soil, foodstuffs, pharmaceuticals) for elemental analysis by ICP, ICP-MS, or AA (Savescu, 2017c). The process necessitates the dissolution of the sample before its introduction into the analyzer (Savescu, 2017c). The sample matrix is subject to acid digestion, a process that serves to disintegrate it, thereby leaving the elements of interest in the solution. After all these operations, the experimental variants are ready for analysis (Savescu, 2017c). The efficiency of microwave electrolytic matrix digestion systems in rapidly digesting a wide variety of sample matrices has been well documented. The analysis of these samples subsequently reveals the analytes of interest in a clear solution (Savescu, 2017c; L'vov, 2005)

The experiment used a 1200 W MARS mineralized microwave EMF system. The MARS EMF system is a multimodal platform equipped with a magnetic stirring plate and a rotor that allows multiple vessels per batch to be processed in parallel (Savescu, 2017b). The experiments were conducted using two different vessels: the first was an HP-500 (Teflon insert - TFA) with an 80-ml vessel volume, a maximum pressure of 350 psi, and a maximum temperature of 210°C; the second was a Chem (borosilicate glass insert) with an 80-ml vessel volume, a maximum pressure of 200 psi, and a maximum temperature of 200°C. Both vessels were based on a fourteen-position rotor (Savescu, 2017b).

The system delivers continuous power between 0 and 1200 W. The temperature is controlled internally by a fiber optic probe in a control reference vessel (Savescu, 2017b).
In summary, 10 g of dry substance tea was measured with analytical precision. For the mineralization step, 10 g of product (black tea), 6 mL of concentrated nitric acid, and 3 mL of 30% hydrogen peroxide were added to each digestion cartridge (Savescu, 2017b).
A digestion cartridge containing no product, only reagents, was used as a control. The AAS method was conducted using a Varian SpectrAA 220Z atomic absorption spectrometer furnace system, equipped with a Varian SpectrAA 220Z autosampler, a Varian GTA 110Z furnace, and a Varian UltrAA. The stages of microwave digestion are described in Table 1.

Table 1. Stages of microwave digestion

Power, W	Time, min	Stirring	Comments
300	4	yes	For cartridge protection
0	1.5	no	To help the sedimentation process on the downside of vessels
400	2.5	yes	
600	2.5	yes	
200	1	no	
800	2.5	yes	
1000	4	yes	
0	10	no	Cooling for opening cartridges

The analysis was facilitated by related Windows interface software (Savescu, 2017a). The results are expressed as the mean ± standard error of the mean (SEM), except some plants with high oil content can be used, which is expressed as the mean ± standard deviation (SD). For better statistical analysis, Kruskal-Wallis two-way ANOVA followed by Dunn's post-hoc test can be used (Savescu, 2017c). All calculations were performed utilizing the ORIGIN PRO 2020 software.

RESULTS AND DISCUSSIONS

When developing innovative black tea supplements, it is important to ensure that the sweeteners used preserve the active forms of flavoproteins and riboflavin.

The results of this stage of the research showed the best sweetening option for the black tea food supplement. The product that was made and chosen to be used (as a raw material) in the next stages of the process had a similar chemical makeup to the unsweetened one, but also much better sensory properties (Savescu, 2017b).
The use of the AAS technique revealed high concentrations of potassium, magnesium, and calcium in the parts of the tea plant used, as shown in Table 2.

Table 2. Cations composition of black tea obtained by AAS technique

Indicator/ Constituent	BLACK TEA	
	Dry matter ppm (mg/kg)*	Watery extract 1:10 ppm (mg/L) Average value
Na^{+}	1012±3.56	153.04
K^{+}	16720.2±3.26	285.21
Ca^{2+}	742±2.46	7.98
Mg^{2+}	462±1.64	7.93
Zn^{2+}	48±2.48	1.79
Mn^{2+}	84.5±1.89	0.23
Fe^{2+}	74.24±1.84	0.68
Al^{3+}	10.42±0.67	0.49
Cu^{2+}	10.46±0.66	0.35
Pb^{2+}	0.036±0.001	missing

In the experimental V_0, the control variant, a peak in NAD^{+} was observed, accompanied by a decrease in tocopherol and a high theobromine content.
When white sugar is added to the experimental variant V_1, minor alterations in pigments and a marginal decline in specific chemicals, notably theobromine, can be discerned (Butoi et al., 2023). Levels of flavoprotein concentrations when using white sugar are relatively low compared to other sweeteners.
The modifying tendency of flavoproteins is also observed when brown sugar is used in V_2, but to a greater extent in terms of protecting oxidized and reduced forms. The concentrations of oxidized and reduced forms of riboflavin are higher in brown sugar than in white sugar, making brown sugar a better option.
Honey used as a sweetener in the V_3 variant showed the highest concentrations of flavoproteins, as well as the highest concentrations of oxidized and reduced forms of riboflavin, as demonstrated in the accompanying graphs.

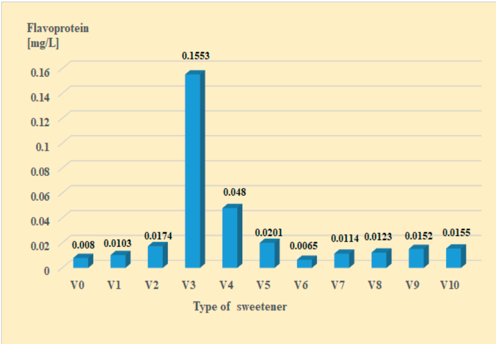

Figure 3. Black tea flavoprotein concentration

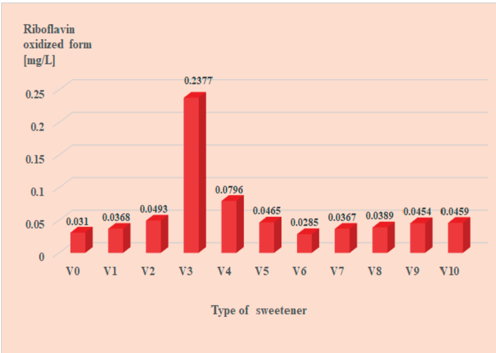

Figure 4. Concentration of the oxidized form of riboflavin in black tea

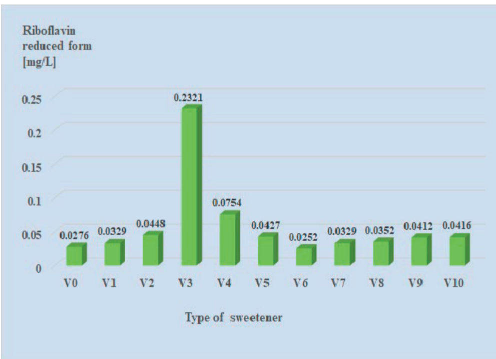

Figure 5. Concentration of the reduced form of riboflavin in black tea

The graphs show that V4 (sweetened with saccharin) has high levels of flavoprotein. The version of the experiment that used saccharin as a sweetener protects oxidized and reduced forms of riboflavin.
Sucrazit (V_5) exerts a significant influence on the concentration of the primary active compounds present in black tea.
The use of Diamond V6 leads to the lowest concentrations of flavoprotein forms, as can be seen in the graph in Figure 3.
Studies have shown that using a mixture of sodium cyclamate and saccharin can help to keep the concentration of specific oxidoreductase coenzymes stable. This also keeps them from losing their strong antioxidant properties (Butoi et al., 2023).
In the case of variant V_7, fructose was utilized. Notably, this does not have a significant effect on the color intensity in the UV and visible range, thereby ensuring the maintenance of the color tone. The levels of oxidized and reduced forms of riboflavin and flavoproteins are maintained at low levels.
The use of sweeteners such as xylitol (V_8), sorbitol (V_9), and stevia sweetener (V_{10}) is mandatory to maintain the levels of oxidized and reduced forms of riboflavin at a consistent level. Sorbitol and stevioside prove similar effects on flavoprotein concentrations.
There is a similar situation regarding the influence of sweeteners on the concentrations of reduced (Figure 4) and oxidized (Figure 5) forms of riboflavin.

CONCLUSIONS

The following conclusions can be drawn from the results:
As evidenced by the obtained results, the honey utilized in the experimental version V_3 induces a considerable influence on black tea flavoproteins. The honey-sweetened (variant V_3) exhibited elevated concentrations of oxidized and reduced forms of riboflavin in black tea.
A similar trend is observed in flavoprotein concentrations. It is evident that V_3, sweetened with honey, shows the highest concentrations of flavoproteins.
When sodium cyclamate was used in combination with saccharin (V_6) it led to the lowest flavoprotein concentrations.
The saccharin used as a sweetener (V_4) in black tea has relatively high flavoprotein concentrations, after honey, which makes it a good sweetener option.

REFERENCES

Belitz, H.-D., Grosch, W., & Schieberle, P. (2009). *Food Chemistry* (4th ed., pp. 95–102). Springer-Verlag Berlin Heidelberg.

Butoi, C., Ionescu, A. I., Schiopu, V., & Popescu, G. (2023). Study on the importance of redox processes catalyzed by NAD- and FMN-dependent oxidoreductases in obtaining a food supplement based on black tea. *Annals of the University of Craiova - Agriculture, Montanology, Cadastre Series, 53*(1). https://doi.org/10.52846/aamc.v53i1.1433

Isenberg, I., & Szent-Györgyi, A. (1958). Free radical formation in riboflavin complexes. *Proceedings of the National Academy of Sciences of the United States of America, 44*(9), 857–862. https://doi.org/10.1073/pnas.44.9.857

L'vov, B. V. (1990). Recent advances in absolute analysis by graphite furnace atomic absorption spectrometry. *Spectrochimica Acta Part B: Atomic Spectroscopy, 45*(7), 633–655. https://doi.org/10.1016/0584-8547(90)80046-L

L'vov, B. V. (2005). Cincizeci de ani de spectrometrie de absorbție atomică. *Journal of Analytical Chemistry, 60*, 382–392. https://doi.org/10.1007/s10809-005-0103-0

Savescu, P. (2016a). Improving the concentrations of bioactive compounds (with antioxidant properties) in alfalfa and corn for their use in food supplements and functional food. *Nano, Bio, and Green-Technologies for a Sustainable Future, SGEM International Conference, Albena, Bulgaria, 6*(1), 583–590.

Savescu, P., Poenaru, M. M., & Iacobescu, F. (2016b). Study regarding the development of organic farming systems in Romania as the basis for obtaining innocuity agricultural raw materials – used in functional food. *Annals of the University of Craiova, Series Agriculture, Montanology, Cadastre, 46*(1), 281–285.

Savescu, P. (2017a). A new approach to study the sweetener's effect on green tea oxidative status. *Revista de Chimie, 68*(2), 294–299. https://revistadechimie.ro/Articles.asp?ID=5439

Savescu, P. (2017b). Comparative study on the effect of sweeteners on the oxidative status of green tea and black tea. *Revista de Chimie, 68*(6), 1406–1412. https://revistadechimie.ro/Articles.asp?ID=5683

Savescu, P. (2017c). A new approach to study the sweetener's effect on Hibiscus tea oxidative status. *International Conference SGEM 2017, Section 25 - Advances in Biotechnology, Albena, Bulgaria, 17*(1), 455–460. https://doi.org/10.5593/sgem2017/61/s25.059

Savescu, P., Badescu, G., Milut, M., Ciobanu, A., Apostol, L., & Vladut, V. (2019). Healthy food – Through innovative technologies. *ISB-INMA-TEH Bucharest*, 516–521.

Savescu, P., Iacobescu, F., & Poenaru, M. M. (2020a). Study on the use of biomaterials as protective membranes for certain functional foods. In S. Piotto et al. (Eds.), *Advances in Bionanomaterials II, Lecture Notes in Bioengineering*. Springer International Publishing. https://doi.org/10.1007/978-3-030-47705-9_48

Savescu, P., Iacobescu, F., & Poenaru, M. M. (2020b). Patent application - OSIM A00745, *Innovative food supplement based on plant products obtained from organic farming*. OSIM, Romania.

Savescu, P. (2021). Natural compounds with antioxidant activity - Used in the design of functional foods. In *Functional Foods - Phytochemicals and Health Promoting Potential*. IntechOpen. DOI: 10.5772/intechopen.97364

Skoog, D. A., Holler, F. J., & Crouch, S. R. (2007). *Principles of Instrumental Analysis* (6th ed., pp. 349–351). Thomson Brooks/Cole.

Tita, O., Lengyel, E., Stegarus, D., Savescu, P., & Ciubara, A. B. (2021). Identification and quantification of valuable compounds in red grape seeds. *Applied Sciences, 11*(11), 5124. https://doi.org/10.3390/app11115124

Toogood, H. S., & Scrutton, N. S. (2020). Thermal, electrochemical, and photochemical reactions involving catalytically versatile ene reductase enzymes. In *The Enzymes* (Vol. 47, pp. 491–515). Academic Press. https://doi.org/10.1016/bs.enz.2020.05.012

Ye, F., Qiao, X., Gui, A., Wang, S., Liu, P., Wang, X., Teng, J., Zheng, L., Feng, L., Han, H., Gao, S., & Zheng, P. (2021). Metabolomics provides a novel interpretation of the changes in main compounds during black tea processing through different drying methods. *Molecules, 26*(21), 6739. https://doi.org/10.3390/molecules26216739

Zhuang, B., Liebl, U., & Vos, M. H. (2022). Flavoprotein photochemistry: Fundamental processes and photocatalytic perspectives. *Journal of Physical Chemistry B, 126*, 3199–3207.