A New Approach to Study the Sweetener's Effect on Green Tea Oxidative Status

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Choosing the best sweetener for the green tea can face/be a big problem due to several composition changes that the green tea registers during the sweetening process, the level of gained toxicity, the different health level of the consumers (especially those with diabetes, cardiovascular or digestive problems, allergies). The additives management (especially for certain categories such as sweeteners, food coloring or preservatives) it's a big issue for both technologists and also any treating physicians. By adding some sweeteners, food coloring or preservatives, food products with a higher sensitive quality but also with a higher risk potential for consumers with certain health problems are obtained. This paper presents a personal method to determine the best natural or synthetic sweetener that can be used without any problems when sweetening the green tea from a free contamination area.

Keywords: green tea, sweeteners, coenzymes (NAD, FMN)

Green tea is a very popular food product that began to be used since 500 years ago in China and 390 years ago in Europe (*green gold that repair all*) [1].

For many Europeans green tea consumption is a new lifestyle, and in Japan has become even more popular than drinking beer or distilled spirits [2].

Green tea is known for its antioxidant effects (preventing accelerate cellular aging by neutralizing free radicals) for the prevention of tumours and cardiovascular diseases (by decreasing the absorption of fatty acids and cholesterol), increase immunity and revive consumer, vitalize them.

To improve sensory characteristics of green tea are commonly used natural sweeteners or synthetic sweeteners and these must be chosen carefully because using them singly or together with other food additives can lead to severe illness [3].

Using natural sweetener (if consumers healthy human) or synthetic sweetener (hypo-caloric, for consumers with diabetes or digestive disorders) induces a number of changes of chemical content to green tea and is therefore very important to know how to choose the best sweetener for this food.

It is very important that the final food (the sweetened green tea) to retain the characteristics of the primary functional food (with very high antioxidant potential) and be non toxic to the consumer. Therefore, were checked (before the experiment) several kinds of green tea and analyzed by atomic absorption spectrometry the concentrations of certain chemical elements (heavy metals and others) - to avoid the use of green tea with traces of pollution.

To check of induced changes in oxidative status of green tea due to the addition of natural or synthetic sweeteners were identified and quantified the changes of concentrations for the oxidized and reduced forms of main coenzyme of oxidoreductases (NAD and FMN).

The study of NAD combinations with various active substances from certain drugs was a basic concern for senior scientists for many years [4].

Nicotinamide adenine dinucleotide (NAD) is a coenzyme found in all living cells. Nicotinamide adenine dinucleotide (NAD) is a coenzyme involved in metabolic redox reactions, a precursor for several cell signaling molecules, and a substrate for protein posttranslational modifications [5].

The compound is a dinucleotide, because it consists of two nucleotides joined through their phosphate groups. One nucleotide contains an adenine base and the other, nicotinamide. Nicotinamide adenine dinucleotide exists in two forms, an oxidized and reduced form abbreviated as NAD^+ and NADH respectively [6].

In metabolism, nicotinamide adenine dinucleotide is involved in redox reactions, carrying electrons from one reaction to another. The coenzyme is, therefore, found in two forms in cells: NAD^+ is an oxidizing agent – it accepts electrons from other molecules and becomes reduced. This reaction forms NADH, which can then be used as a reducing agent to donate electrons. [7] These electron transfer reactions are the main function of NAD. However, it is also used in other cellular processes, the most notable one being a substrate of enzymes that add or remove chemical groups from proteins, in posttranslational modifications. Because of the importance of these functions, the enzymes involved in NAD metabolism are targets for drug discovery [8].

In metabolism, the compound accepts or donates electrons in redox reactions [9].

Both NAD^+ and NADH strongly absorb ultraviolet light because of the adenine. From literature, the peak absorption of NAD^+ is at a wavelength of 259 nanometers (nm), with an extinction coefficient of 16,900 $M^{-1}cm^{-1}$. [10] NADH also absorbs at higher wavelengths, with a second peak in UV absorption at 339 nm with an extinction coefficient of 6,220 $M^{-1}cm^{-1}$ [11]. This difference in the ultraviolet absorption spectra between the oxidized and reduced forms of the coenzymes at higher wavelengths makes it simple to measure the conversion of one to another in enzyme assays – by measuring the amount of UV absorption at 340 nm using a spectrophotometer [12].

The main role of NAD^+ in metabolism is the transfer of electrons from one molecule to another. Reactions of this type are catalyzed by a large group of enzymes (the oxidoreductases). The correct names for these enzymes contain the names of both their substrates: for example NADH-ubiquinone oxidoreductase catalyzes

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the oxidation of NADH by coenzyme Q. However, these enzymes are also referred to as *dehydrogenases* or *reductases*, with NADH-ubiquinone oxidoreductase commonly being called *NADH dehydro-genase* or sometimes *coenzyme Q reductase* [13].

These oxidoreductases are very active in foods that are rich in antioxidant – like as green tea.

The redox reactions catalyzed by oxidoreductases are vital in all parts of metabolism, but one particularly important area where these reactions occur is in the release of energy from nutrients. Here, reduced compounds such as glucose and fatty acids are oxidized, thereby releasing energy. This energy is transferred to NAD^+ by reduction to NADH, as part of beta oxidation, glycolysis, and the citric acid cycle [14].

The oxidoreductases activity may be influenced by the active substances of sweeteners that are able to develop some fields of different energies.

It is therefore very important the study of sweeteners action in green tea through analyzing the concentrations of reduced and oxidized forms of NAD and FMN (to know the effects on aerobic and anaerobic environment of tea).

A similar behaviour has been studied in an applied research on the metabolism of the *Saccharomyces cerevisiae* yeast [15].

Since both the oxidized and reduced forms of nicotinamide adenine dinucleotide are used in main sets of reactions, the cell maintains significant concentrations of both NAD^+ and NADH, with the high NAD^+/NADH ratio allowing this coenzyme to act as both an oxidizing and a reducing agent [16].

Flavin mononucleotide (FMN), or riboflavin-52 - phosphate, is a bio-molecule produced from riboflavin (vitamin B_2) by the enzyme riboflavin kinase and functions as prosthetic group of various oxidoreductases including NADH dehydrogenase as well as cofactor in biological blue-light photo receptors. During the catalytic cycle, a reversible interconversion of the oxidized (FMN), semiquinone ($FMNH^{\bullet}$) and reduced ($FMNH_2$) forms occurs in the various oxidoreductases [17]. FMN is a stronger oxidizing agent than NAD and is particularly useful because it can take part in both one- and two-electron transfers. In its role as blue-light photo receptor, (oxidized) FMN stands out from the 'conventional' photo receptors as the signalling state and not an E/Z isomerisation [17]

It is the principal form in which riboflavin is found in cells and tissues. It requires more energy to produce, but is more soluble than riboflavin [18]

Flavin mononucleotide is a coenzyme for a number of oxidative enzymes including NADH Dehydrogenase. It is the principal form in which riboflavin is found in cells and tissues. The development of the spectral technique furthermore opens new horizons for studying the dynamics of redox proteins [19].

Experimental part

Microwave digestion -Method

In order to determine correctly the influence of certain natural and synthetic sweeteners on the level of oxidative status green tea and for sample preparation were performed chemical analysis for the determination of heavy metals and minerals from several varieties of green tea.

For experiment was used tea which showed no traces of pollution or contamination and therefore could not create pre-catalytic oxidation conditions.

In the first phase sample preparation for atomic absorption spectrometry was used mineralization (microwave digestion). Microwave digestion is used to prepare samples of all types (rocks, plant, soil, food, pharmaceuticals, plastics, metals) for elemental analysis by ICP, ICP-MS, or AA, which require the sample to be in the form of a solution in order to introduce it into the analyzer. Acid digestion is employed to break down the sample matrix leaving the elements of interest in solution and ready for analysis. CEM microwave digestion systems rapidly break down a wide variety of sample matrices leaving behind a clear solution containing the analytes of interest.

Microwave technology has become a common tool for chemical synthesis both in academia and industry. Compared to conventional means of synthesis, the advantages of heating with a microwave include:

- faster reaction times;
- greater yields;
- improved purity;
- better reproducibility;
- enhanced reaction control.

The microwave accelerated reaction system is designed for digesting, dissolving, hydrolyzing a wide variety of materials in a laboratory setting. The system uses microwave energy to heat samples in polar or ionic solutions rapidly and at elevated pressures. Its main purpose is for preparing samples for analysis by atomic absorption (AA).

For this purpose it used a CEM Mars system of microwave mineralized, 1200W. The MARS system of CEM is a multi-mode platform equipped with a magnetic stirring plate and a rotor that allows the parallel processing of several vessels per batch. We used the HP-500 (Teflon (TFA) insert) (vessel volume 80 mL, max pressure 350 psi, max temperature 210°C) and Greenchem (glass (borosilicate) insert) (vessel volume 80 mL, max pressure 200 psi, max temperature 200°C) vessel assembly types both based on a fourteen positions rotor. The system delivers a continuous power output between 0 and 1200 W. Temperature is controlled internally by fibber optic probe in one control reference vessel. On-line pressure monitoring of the reference vessel is also provided. All rotor segments are protected by a vent nut that contains a rupture membrane. Additionally, the system is equipped with a solvent sensor detector safety feature. Cooling down of the rotor segments to room temperature is done by an air flow provided by the exhaust fan.

Method: Briefly, was weighed with analytical precision 10 g dry substance (d.s.). For the mineralization was added to each digestion cartridge 10.0000 g product (green tea), 6 mL of concentrated nitric acid and 3 mL of 30% hydrogen peroxide. For blank was used one digestion cartridge without product, just reagents. The method is summarized in table 1.

Atomic adsorption and UV-Vis spectroscopy

For AAS method was used one Varian SpectrAA 220Z Atomic Absorption Spectrometer Furnace System with Varian SpectrAA 220Z Auto Sampler , Varian GTA 110Z Furnace, Varian UltrAA and afferent Windows interface software.

For to obtain of the witness experimental variant (unsweetened) it has been used green tea packed in little special envelopes and that was boiled and adequate separated.

One Chinese green tea (High Green Tea made by Bi Luo Chun and imported in Romania by SC Plant LLC Bucureºti) has been used as basis for experiment. It was used a tablespoon of green tea in 250 mL hot water in a steady 3 min infusion.

Power ,W	Time, min	Agitation	Comment
300	5	Yes	For protect of cartridges
0	2	No	For helping the sedimentation process in to blase of cartridge
400	3	Yes	
600	3	Yes	
200	1	No	
800	3	Yes	
1000	5	Yes	
0	10	No	Cooling for open the cartridges

Table 1
THE MICROWAVE DIGESTION STEPS

The sweetening operation was carried out under the same conditions of temperature and pressure. To avoid errors of analysis were verified areas of maximum absorbance (where molecular absorption spectra recorded a maximum peak) for NAD, NADH + H^+, FMN; FMNH + H^+ and used the Unique Addition method and standards Pure Analysis substances for each compound using as the baseline unsweetened green tea.

In the Unique Addition method were used NAD Pure Analysis substance from Sigma Aldrich (20mg/vial type N8410-15VL, stored at -20°C) and Flavin Mononucleotide FMN Pure Analysis from Sigma Aldrich (100 mg/pck tip CDS020791). β-Nicotinamide adenine dinucleotide (NAD+) and β-Nicotinamide adenine dinucleotide, reduced (NADH) comprise a coenzyme redox pair (NAD+:NADH) involved in a wide range of enzyme catalyzed oxidation reduction reactions.

In order to achieve a correct correlation have been measured the variation of *p*H and redox potential to experimental variants – follow the applied sweetening operation. Both results achieved by UV-Vis spectral analysis and the results of the variation in *p*H and Eh (redox potential) were subjected to statistical analysis mathematical correct interpretation of the results.

After being boiled (50-60g green tea / L water) and cooled, the obtained drink was decanted and filtered (through a porous cellulosic material). After filtration task, the green tea was centrifugal separated into a performance centrifuge *Sygma* type, at a 4400 rot/min during 10 min.

After the centrifugal separation had been picked a median sample of 50 mL green tea drink that was diluted; this variant being the unsweetened reference one.

At this reference sample it had been added the principal sweeteners admitted in Romania: naturals or synthetics-obtaining other 7 experimental variants:

V1- unsweetened green tea (GT = reference sample);
V2- green tea with sugar sweetener;
V3- green tea with saccharine sweetener;
V4- green tea with Aspartame sweetener (from *Equal* product);
V5- green tea with Edulciclam sweetener;
V6- green tea with Zuckli sweetener;
V7 - green tea with Sucrazit sweetener;
V8 - green tea with honey sweetener.

The saccharine had been produced by SICOMED (19 mg saccharine/tablet)-and had been added into boiled tea; the Aspartame was by NUTRASWEET (Equal Brand Sweetener) (90 mg Equal/tablet), Edulciclame (100 mg Natrium Cyclamate/tablet) was from S.C.ARMEDICA S.A., Zuckli (40 mg Cyclamate + 4mg Saccharine/ tablet), had been produced by BERLINER CHEMIE, Sucrazit (Natrium bicarbonate 59.52%, Saccharine 23.81%, fumaric acid 16.67% for one tablet) had been produced by BISCOL CO LTD (from Elite).

The experimental samples were spectrophotometer to a digital high performance spectrophotometer UV-Vis *T92+* type from PG Instruments, in the nearly UV range (190-400 nm), the visible range (400-700 nm) and nearly IR range (700-900 nm). The T92+ is a high performance double beam spectrophotometer with a variable spectral bandwidth from 0.1-5nm, selected by a continuous variable slit and Photometric Range -4.0 to 4.0Abs and Photometric Mode: Transmission, Absorption, Reflectance, Energy & Concentration. At 325 nm was automat interchanged the Deuterium lamp with a Wolfram one (was selectable within the working range of light source).

For minimise analytical errors it used a thermostatic system controlled in all mean UV-Vis-IR controlled by a manual re-tracking (at accuracy of ±0.3nm (Automatic Wavelength Correction).

During the analysis for the experimental variants it has been taken all the treatments, for having a minimal temperature changes at the maxim limit of the interfering substances influence, the assure the optimal needed conditions for a average analytical errors limits. During the analysis and the interpretation of the results it has been used from the utilitarian packet MS Office 2000: MS Word2000 and MS Excel 2000. The statistical analysis for data obtaining has been effectuated with the IBM SPSS Statistics 24.0.0 (SPSS Sample Power) - statistical utility software for Windows.

Results and discussions

The results in determining the heavy metal and mineral elements contain from the green tea used for the sweetener effect on NAD and FMN coenzymes check are represented in the table 2.

In order to observe how high element content is in the tea type chosen for sweeting test, these elements were quantified also from the chemical tries on aqueous extract (1:10).

It has been controlled experimentally the influence of the sweeteners added in the green tea drink, evident the Absorption in the nearly UV range, Vis and near IR rage too.

After the process of spectrophotometry on UV-Vis-IR ranges, It had been obtaining more than 1100 pairs of data that were statistic prepared resulting after the statistic data analysis that the sweetening variant with Aspartame (V4)

Indicator	Dry Matter ppm (mg/kg)	Aqueous extract 1:10 ppm (mg/L)	Comment
Na^{+}	1020	107.4	
K^{+}	1398	99.9	
Ca^{2+}	246	2.64	
Mg^{2+}	120	3.075	
Zn^{2+}	26.4	0.93	
Mn^{2+}	259.5	1.4325	
Fe^{2+}	9.225	0.12	
Al^{3+}	6.975	0.4725	
Cu^{2+}	5.475	0.27	
Pb^{2+}	0.0225	lack	

Table 2
ATOMIC ABSORPTION SPECTROSCOPY FOR GREEN TEA BASE LINE

was the best for the green tea: the Pearson correlation being the only two of 1,000 in the follow pair of variants Unsweetened /with Equal and Unsweetened /with sugar (comparing to the other pairs) but the partial correlation coefficients showed 1,000 for pair of variants Unsweetened /with Equal (V1/V4) and 0.9996 for pair of variants Unsweetened /with sugar (V1/V2). For compares, the partial correlation coefficients was 0.9922 for pair Unsweetened /with saccharine, 0.9984 for pair Unsweetened /with Edulciclam, 0.9988 for Unsweetened /with Zuckli, 0.9970 for pair Unsweetened /with Sucrazit; the Pearson Correlation was 0.999 for pairs Unsweetened /with Zuckli and Unsweetened /with honey, 0.998 for pair Unsweetened /with Edulciclam, 0.997 for pair Unsweetened /with Sucrazit and 0.992 for pair Unsweetened /with Saccharine.

For establish the data frequencies the total work average cases were selected in to 211 cases and computable first lags: 209, the processor had been accepted 210 valid average pairs Absorption/ wave-length (missing one value).

After analyse the paired samples test, Paired Differences the Pair3 (Unsweetened /with Aspartame) show 0.1338 Standard Deviation (the littlest standard deviation reported by natural variant) and Pair 1 (Unsweetened /with sugar) show 0. 2896 Standard Deviation (table 3).

Partial correlation coefficients stabilization has a significant importance when identifying the best experimental types.

In identifying of the best experimental variant, the primary role have the partial correlation coeficients.

According to the obtained results, the highest value of FMN oxidised forms concentration is registered for

Table 3
PAIRED SAMPLES TEST

		Paired Differences					t
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
					Lower	Upper	
Pair 1	Unsweet. / With Sugar	-0.17741	0.28964	0.0199	-0.21681	-0.13801	-8.877
Pair 2	Unsweet. / With Saccharine	3.40233	7.65548	0.5283	2.36091	4.44377	6.440
Pair 3	Unsweet. / With Aspartame	0.04415	0.13382	0.0092	0.02595	0.06236	4.781
Pair 4	Unsweet. / With EDULCICL	1.13491	2.27123	0.15673	0.82594	1.44388	7.241
Pair 5	Unsweet. / With ZUCKLI	0.84424	2.09939	0.14487	0.55865	1.12984	5.828
Pair 6	Unsweet. / With SUCRAZIT	1.65838	3.89809	0.26899	1.12809	2.18866	6.165
Pair 7	Unsweet. / With Honey	1.64866	3.49463	0.24115	1.17325	2.12406	6.837

Table 4

TEST OF ASSOCIATION (THE PAIRS)

Pair	R	R Squared	Eta	Eta Squared
1	2	3	4	5
With Sugar / Unsweet.	1.000	0.999	1.000	1.000
With Saccharine / Unsweet.	0.992	0.984	1.000	1.000
With ASPARTAME / Unsweet.	0.996	1.000	1.000	1.000
With EDULCICL / Unsweet.	0.998	0.997	1.000	1.000
With ZUCKLI / Unsweet.	0.998	0.998	1.000	1.000
With SUCRAZIT / Unsweet.	0.997	0.994	1.000	1.000
With Honey / Unsweet.	0.999	0.997	1.000	1.000

Table 5

PARTIAL CORRELATION COEFFICIENTS

	With Sugar	With Saccha-rine	With EQUAL	With EDULCI-CLAM	With ZUCKLI	With SUCRAZIT
Un-sweet.	0.9996	0.9922	1.0000	0.9984	0.9988	0.9970

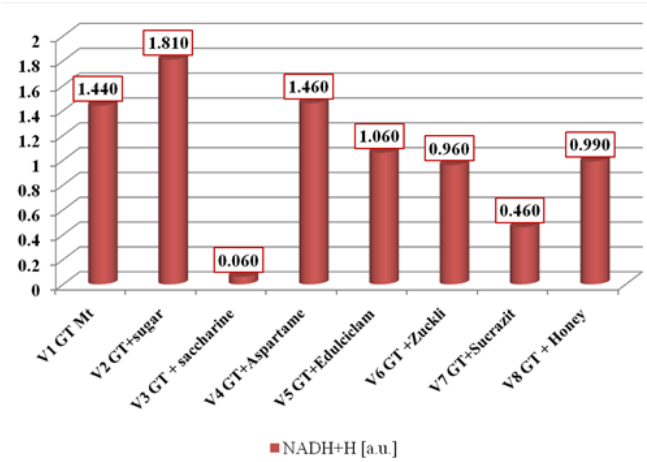

Fig.1. The NADH+ H^+ molecular absorption of experimental variants

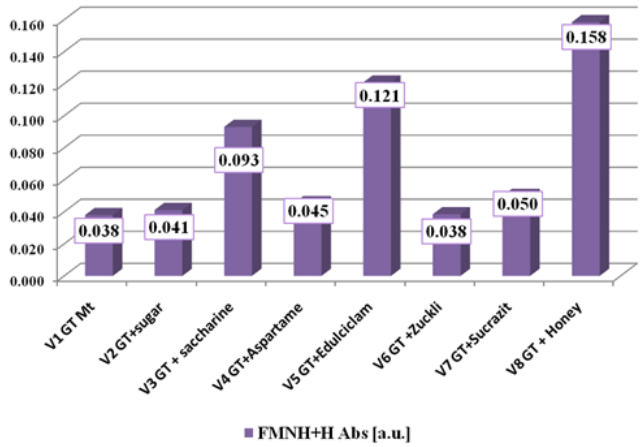

Fig. 2. The FMNH+ H^+ molecular absorption of experimental variants

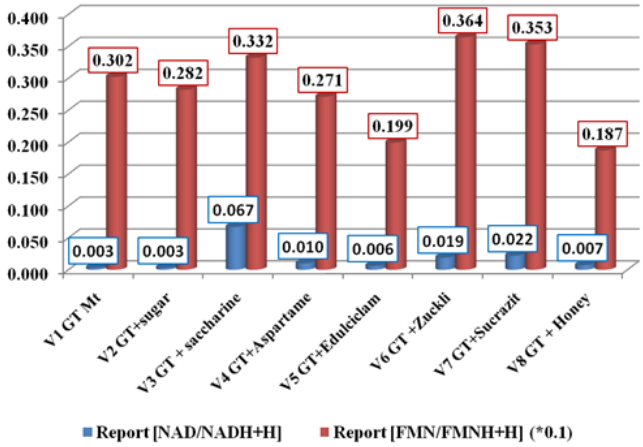

Fig. 3. The Reports of concentration for the oxidised and reduced forms of NAD and FMN for experimental variants

experimental variant V3 – sweetened with Sucrazit/ saccharine (0.309 a.u.). The highest activity of FMN addicted enzymes oxidised forms is registered for the sweetened with Sucrazit/saccharine variant, the honey sweetened variant (0.297 a.u.), Edulciclam sweetened variant (0.241 a.u.). Analysing the molecular spectres curves for sweetened variants we saw that FMN addicted enzymes are simultaneous active at the sweetening process, oxidised and reduced forms concentrations of FMN coenzymes rising above the values of the unsweetened variants.

As per the graphic in figure 1 result, the highest concentrations of reduced forms of FMN coenzymes from green tea were registered at experimental variants V8 (where honey was used as sweetener) and V5 (where Na cyclamate was used as sweetener). In the same time the lowest concentration value for oxidised forms of FMN coenzymes was registered at the same experimental variants.

The highest value of the report between form concentrations (FMN)/ (FMNH+H^+) is registered at variant V6 (Zuckli sweetened green tea), V7 (Sucrazit sweetened green tea) and V3 (Saccharine sweetened green tea); we

can surely affirm that saccharine generates a major oxidising effect in green tea especially close to the separation limit between air and tea, at the liquid surface (when it is either sole used or in association with others).

The ratio of oxidized to reduced molecules, [Ox]/ [Red] is equivalent to the probability of being oxidized (giving electrons) over the probability of being reduced (taking electrons).

As the system redox potential is higher, the system is more oxidised and the opposite is also valid; this is why we can affirm that saccharine sweetening favours the appearance of the most oxidised system for the green tea so this sweetening variant can be considered the less recommended experimental variant. From figure 3 we can clearly observe this aspect; the reports between oxidised and reduced forms of NAD and FMN coenzymes at Saccharine sweetened variant (V3) having the highest values, the sweetening system will be the most oxidised.

The best reports values for oxidised and reduced forms of NAD and FMN coenzymes were registered for the Edulciclam sweetened variant (V5) and honey sweetened variant (V8), with the lowest redox potential values and the most reducing sweetening systems.

Conclusions

Follow this very low cost method I can appreciate the best sweetening variant for the green tea (and through extrapolate for more other product receipt).

Analysing the molecular spectres curves for sweetened variants we saw that FMN addicted enzymes are simultaneous active at the sweetening process, oxidised and reduced forms concentrations of FMN coenzymes rising above the values of the unsweetened variants.

The saccharine generates a major oxidising effect in green tea especially close to the separation limit between air and tea, at the liquid surface (when it is either sole used or in association with others).

The best reports values for oxidised and reduced forms of NAD and FMN coenzymes were registered for the Edulciclam sweetened variant (V5) and honey sweetened variant (V8).

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