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A NOVEL METHOD AND VALIDATION FOR THE QUANTIFICATION OF SACCHARINE AND ASPARTAME IN THE LOCAL MARKETED PRODUCTS

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Keywords:

Artificial sweetener, Saccharin, Validation, Obesity, Aspartame, Diabetes, UV visible spectroscopy

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ABSTRACT: Artificial sweeteners are non-nutritive sugar substitutes designed to provide sweetness without the calorie content of sugar. Examples like Saccharin, Aspartame, Neotame Acesulfame K and they are used in producing a low- or no-calorie diet to lose weight. In the last few years, they have be used in most of the products. Now a days artificial sweeteners are used because they have no calories and pose no risk of obesity. It provides the detailed description of some of the artificial sweeteners and their history, uses and adverse effects. They are often used in food and beverages as alternatives to sugar, providing individuals pursuing to reduce their sugar intake. The artificial sweeteners have an important impact on people who have diabetes or overweight. Validation was performed to know the Linearity, Precision, Ruggedness, Robustness, Limit of detection, Limit of quantification. A simple method is used to determine the artificial sweeteners like saccharin and Aspartame based on UV visible spectrophotometric method and this article provides the analysis of Saccharin and Aspartame and including their validation parameters. For Saccharin a simple method developed by using simple distilled water and the linear responses were recorded in the concentration rangesof10µg/ml-90µg/ml with a good correlation coefficient (r^2) of 0.9922, and for Aspartame a chromogenic method has been developed that is by using Ninhydrin Reagent, according to the ICH Q2(R2) guidelines and the linear responses were recorded in the concentration ranges of 10µg/ml-175µg/ml with a good correlation coefficient (r^2) of 0.9971, The precision was found to be within the acceptable limits (%RSD ≤ 2) for all the methods.

INTRODUCTION: Artificial sweeteners are the synthetic sugar substitutes used to sweeten the foods and beverages without the calories of natural sugars. They are significantly sweeter than the sugar, and while they can provide sweetness, they don't contribute to increased blood sugar levels [1].

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Common artificial sweeteners include Saccharin, aspartame, sucralose, stevia. These substitutes are used in various products to cater to individuals seeking reduced-calorie or sugar free options due to dietary restrictions or health concerns.

Artificial sweeteners are the ingredients added to yogurt, foods, candy, jams, soft drinks, beverages, to provide sweetness without adding a calorie [2].

Saccharin: Saccharin is an artificial sweetener which has been used for over a century as a sugar substitute. Here's a more in-depth look at saccharin:

TABLE 1: PROFILE OF SACCHARIN

Brand Name	Sweet and Low, Necta Sweet
Emperical Formula	C7H5NO3S
INS no	E954
Solubility	Water
Melting Point	228.8 to 229.7C
Appearance	White Crystaline solid

Chemical Structure: Saccharin has the chemical formula C7H5NO3S and is a white, crystalline powder. It is created with a chemical synthesis process from petroleum-based chemicals.

Sweetness: Saccharin is well-known for its extreme sweetness. It is 200 to 700 times tastier than sucrose (table sugar), depending on concentration and individual taste sensibility.

History: Constantin Fahlberg, a researcher at Johns Hopkins University, developed saccharin in 1879. It was accidentally synthesized when Fahlberg noticed a sweet taste on his hands after working with coal tar derivatives [3]. This chance discovery led to the development of saccharin as a sugar substitute.

Usage: Saccharin is a sugar substitute found in a variety of beverages and food items. It is frequently used in low-calorie or sugar-free kinds of soft drinks, canned fruits, jams and other processed foods [4]. Some tabletop sweeteners also contain it.

Safety: Saccharin has been the subject of numerous safety concerns over the years. It was linked to bladder cancer in laboratory rats in the 1970s, prompting the addition of warning labels to products containing saccharin. However, subsequent research and re-evaluations have demonstrated that the cancer risk in humans is much lower than previously [5] thoughts, and several nations have since removed these warning labels.

Market: Saccharin is sold under a variety of different brand names and remains in use as a sugar substitute in a variety of products around the world. Because of its stability, it keeps on being an easily available and used artificial sweeteners, particularly in products requiring a long shelf life. People should be aware of any safety guidelines and recommendations regarding saccharin consumption given by the regulatory authorities in their respective countries [6]. Furthermore, some people may be sensitive to saccharin's aftertaste, as it may possess a somewhat bitter or metallic flavor in high concentrations.

Adverse Effects:

Bladder Cancer (in Rats): Early studies linked high doses of saccharin to an increased risk of bladder cancer in laboratory rats. However, further research in human did not conclusively establish in similar risk, and regulatory agencies consider it safe for human consumption within acceptable limits.

Allergic Reactions: Some individuals may experience allergic responses, such as rashes, itching, or difficulty breathing after consuming saccharin.

Gastrointestinal Distress: In some cases, saccharin can lead to digestive issues, including diarrhoea, bloating, or gas especially in individuals sensitive to artificial sweeteners.

Migraines and Headaches: Sensitivity to saccharin might trigger migraines or headaches in some people.

Potential Blood Sugar and Insulin Effects: While saccharin itself doesn't affect blood sugar or insulin levels, some research suggests it might interfere with the body ability to regulate glucose, potentially impacting blood sugar control [7].

Aspartame: Aspartame is a low-calorie synthetic sugar substitute that is commonly used to replace sugar in a variety of food and beverage products. Here's a quick primer on aspartame [8].

TABLE 2: PROFILE OF ASPARTAME

Brand Name	Equal (or) Nutra Sweet
Emperical Formula	C14H18N2O5
INS no	E951
Solubility	Water
Boiling Point	228.8C
Appearance	White crystalline powder

Chemical Structure: Aspartame is a kind of dipeptide that contains two amino acids in it such as aspartic acid and phenylalanine, combined by a methyl ester. C14H18N2O5 is its chemical formula [9].

Sweetness: Aspartame is extremely sweet, 200 times sweeter than sucrose (also known as table sugar). Because of its intense sweetness, very little

is needed to reach the desired level of sweetness in beverages and [10] food.

Usage: Aspartame is commonly found in free of sugar and low-calorie foods. It's common in diet beverages, sugar-free gum, sugar-free desserts[11], along with a variety of processed foods labelled "light," "diet," or "sugar-free."

Heat Sensitivity: Aspartame is heat sensitive and can degrade when subjected to high temperatures[12]. As a result, it is not appropriate for baking or cooking and is usually incorporated into products after they have been cooked or baked.

Metabolism: In the body, aspartame is disassembled into amino acids that are the compounds aspartic acid & phenylalanine, along with methanol. Which People with phenylketonuria (PKU), a very uncommon genetic disorder, must carefully monitor their phenylalanine intake because they cannot properly metabolize it [13].

Safety: Aspartame has been subjected to extensive testing for safety, and now it is approved for use as a food additive in a number of countries, which includes the United States and Europe [14].

Aspartame has acceptable daily intake levels established by regulatory authorities such as the United States Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) [15].

Controversy: Aspartame has been the subject of some controversy regarding health concerns, but a majority of scientific studies and regulatory agencies have determined that it is safe to be consumed within certain levels [16]. Individuals with PKU should be mindful of their phenylalanine intake, as too much aspartame can be harmful.

Overall, aspartame is a frequently employed artificial sweetener that offers sweetness with few calories, making it an appealing option for people looking to decrease their consumption of sugar and calorie intake [17].

However, as with all food additives, it must be consumed in small amounts, and people with particular medical conditions should consult with healthcare professionals for nutritional advice.

Adverse Effects:

Headaches: Some people have reported headaches after consuming aspartame-containing products. While the cause of this phenomenon is unknown, it is frequently referred to as "aspartame-induced headaches."

Gastrointestinal Distress: After consuming aspartame-containing products, some people might experience digestive problems such as gas, bloating, or diarrhoea. These side effects are typically mild and transient [18].

Altered Taste: For some people, aspartame may leave a slight aftertaste or alter their taste perception, which may affect the tasting experience of certain foods and beverages.

It's important to note that a significant amount of scientific research and regulatory bodies, includes the Food and Drug Administration of the United States (FDA) and the European Food Safety Authority (EFSA), have come to the conclusion that aspartame is safe for human consumption within developed ADI levels. The acceptable daily intake (ADI) for aspartame has been set far below the levels commonly found in the diet [19]. Individual reactions to aspartame, like any other food additive, can vary, and some people may be more sensitive to its affects than others. Individuals with specific health conditions, such as PKU, as well should follow dietary recommendations provided by healthcare professionals. If you are concerned about consuming aspartame or are experiencing adverse effects that you believe are related to it, you should seek personalized advice from a healthcare provider [20].

MATERIALS AND METHODS:

Active Pharmaceutical Ingredient: Saccharin and Aspartame were obtained by SDFCL limited, in Hyderabad, as a gift sample.

Reagents and Chemicals: Saccharin Powder, Sulphuric Acid, Diethyl ether, Sodium hydrogen carbonate, Isoamyl alcohol, Formic Acid, Bromine water, Potassium Iodide, Hydrochloric Acid, Distilled water, Cetyl trimethyl ammonium bromide, Carrez Solution 1, Carrez Solution 2, Acetate buffer, Ethanol, Acetone, Ninhydrin, Sodium Acetate, Acetic acid.

Preparation of Reagents:

Formic Acid: 25ml of formic acid was dissolved in 25ml of water was to prepare 50% v/v**.**

Potassium Iodide: To prepare 1% w/v of potassium iodide 1g was dissolved in 100ml of water.

Sodium Hydrogen Carbonate: 2 grams of $NaHCo_3$ was dissolved in 100ml of water to prepare 2% solution.

CTAB: 1gram of CTAB was dissolved in 50 ml of water.

5% HCl: 4ml of HCl was dissolved in 6ml of water.

Carrez Solution 1: 1.5 grams of potassium hexacyanoferrate was dissolved in10 ml of distilled water**.**

Carrez Solution 2: Dissolve 3 grams of Zinc Sulfate in 10 mlof distilled water.

Acetate Buffer: Prepare 400ml of distilled water in a beaker, add 2.8 grams of sodium acetate, add 1ml of acetic acid and adjust the solution to desired pH using 10N HCl and add the distilled water up to 500ml.

Ninhydrin Reagent: By dissolving 10mg of Ninhydrin in 20 ml of Acetone.

METHOD-1

Diluents: Distilled water.

Standard Stock Solution: Weigh out 10 mg of pure Saccharin in a 10 ml volumetric flask and then use distilled water to make up and perform the calibration curve.

Dilutions: Using distilled water as a diluent for necessary dilution from standard stock solution of drug sample λ_{max} of the drugsample were scanned with the help pf uv-visible spectrophotometer against blank using water. The absorption curve shows characteristic λ_{max} at 352nm for Saccharin.

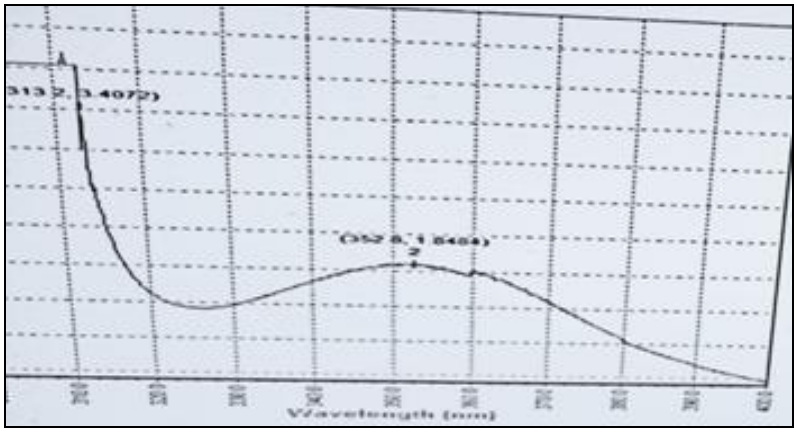

FIG. 1: SPECTRUM OF SACCHARIN WITH DISTILLED WATER

Calibration Curve: Distilled water is used as a solvent for the preparation of calibration curve at λ_{max} 352nm by using ELICO UV-Visible Spectrophotometer.

Working Standard Solution: It is done by weighing to prepare the standard stock sample accurately 10mg of Saccharin in 10 ml volumetric flask and makeup with distilled water. Then 10µg/ml -90µg/ml were prepared by using distilled water as a diluent for serial dilutions and the absorbance were taken at λ_{max}352nm against the blank. The concentration vs. absorbance data yields a standard calibration curve that is reported.

Extraction Procedure: By weighing accurately10 ml of sample was introduced into a separating funnel., along with 1 ml of 10% sulphuric acid & the mixture was extracted with 12 ml of Diethyl ether.

Then the lower aqueous layer was discarded, and the upper ether layer was extracted with 4ml of 2% Sodium hydrogen carbonate solution before discarding. Before extracting with 10ml of diethyl ether, the aqueous layer was acidified with 2ml of 5% HCl. Ethereal extract evaporated in a hot water bath. Then the residue was completely dissolved in 10ml of water and aliquots were analysed.

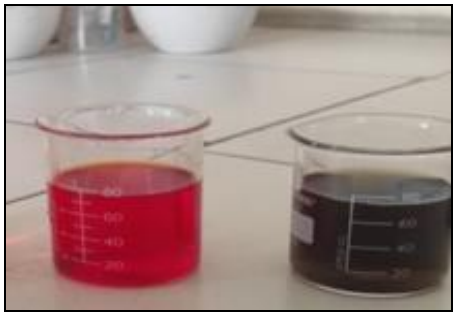

FIG. 2: SAMPLES AFTER EXTRACTION

Method 2:

Diluents: Distilled water, Ninhydrin reagent.

Standard Stock Solution: Weigh 10 mg of pure Aspartame in a 10 ml volumetric flask and then make up with distilled water and perform the calibration curve.

Dilutions: Using distilled water and Ninhydrin reagent as a diluent for necessary dilution from standard stock solution of drug sample λ_{max} of the drugsample were scanned with the help pf uv-visible spectrophotometer against blank using water. The absorption curve shows characteristic λ_{max} at 570nm for Saccharin.

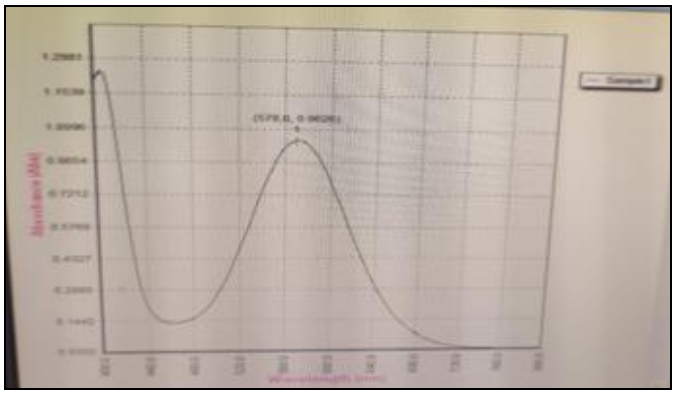

FIG. 3: SPECTRUM OF ASPARTAME WITH 4ML OF NINHYDRIN

Calibration Curve: Ninhydrin reagent as a solvent for the preparation of calibration curve at λ_{max} 570nm using ELICO UV Visible Spectrophotometer.

Working Standard Solution: It is done to prepare the standard stock sample by weighing accurately 10mg of Aspartame solution in 10ml volumetric flask and makeup with distilled water. Then 10µg/ml -175µg/ml were prepared by adding 4ml Ninhydrin reagent for serial dilutions and the absorbance were taken at λ_{max} 570nm against the blank. The concentration vs. absorbance data yields a standard calibration curve that is reported.

Extraction Procedure: In a 100ml volumetric flask, weigh 20 grams of homogenized sample, and place the flask in an ultrasonic bath 20 minutes at 40 degrees Celsius after adding 60ml of water. Since the aspartame has degraded, temperature settings should not exceed 40 degrees Celsius. Allow it to cool to room temperature. Then add 2ml of Carrez solution1 and 2ml of Carrez solution 2, Shake vigorously and set aside for 20 minutes. After the solution is diluted up to the mark and filter the solution. If in case any amount of fat-free insoluble matter in the sample solution exceeds 3 grams, then centrifuge it for 10 minutes at 1400rpm. If any cloudy particles remain in the sample solution, wash it with water and centrifuge it.

FIG. 4: SAMPLE AFTER EXTRACTION

RESULTS AND DISCUSSION:

Method 1: Saccharin: In the process, we have developed and validated an UV-Visible spectroscopic method. The procedures were approved in accordance with ICH guidelines. The linearity was 10-90µg/ml for UV Spectroscopic method individually, demonstrating the R^2 of 0.9922. The UV spectroscopic technique was found to be linear, accurate, robust, and rugged.

Linearity: Ability to obtain test results which are directly proportional to the concentration of the analyte in the sample.

Procedure: For preparing serial dilutions pipette out 1, 2, 3, 4, 5, 6, 7, 8, 9 ml from 100ppm solution and transfer to separate 10mlvolumetric flask and makeup with diluent to yield 10, 20, 30, 40, 50, 60, 70, 80, 90 ppm solution respectively.

TABLE 3: LINEARITY DATA OF SACCHARIN WITH DISTILLED WATER

S. no.	Concentration	Absorbance
1	10	0.0014
2	20	0.1054
3	30	0.2497
4	40	0.4239
5	50	0.5998
6	60	0.7993
7	70	1.0412
8	80	1.2315
9	90	1.3982

FIG. 5: CALIBRATION CURVE OF SACCHARIN WITH DISTILLED WATER

Precision: 6 replicates of 40ppm Standard solutions of Saccharin absorbance was noted at wavelength 352 nm. % RSD was calculated and obtained results are within the limits as per ICH Guidelines.

TABLE 4: RESULTS OF PRECISION DATA OF SACCHARIN

Concentration (ppm)	Absorbance
40	0.4239
40	0.4272
40	0.4234
40	0.4263
40	0.4284
40	0.4278
Mean	0.426166667
SD	0.002075
%RSD	0.486958

Robustness: It is a measure of its capacity to remain unaffected by small, but deliberate changes in method parameters.

It was calculated by measuring the absorbance of sample at different wavelength 351nm, 352nm and 353nm.

The Percentage Relative Standard Deviation (%RSD) was calculated and tabulated. The Percentage Relative Standard Deviation was calculated and results are within the limits as per ICH Guidelines.

TABLE 5: RESULTS OF ROBUSTNESS DATA OF SACCHARIN

Concentration (ppm)	Absorbance at 351	Absorbance at 352	Absorbance at 353
40	0.3262	0.4239	0.5263
40	0.3212	0.4272	0.5214
40	0.3232	0.4234	0.5232
40	0.3298	0.4263	0.5299
40	0.3283	0.4284	0.5283
40	0.3248	0.4278	0.5247
Mean	0.325583333	0.426166667	0.525633333
SD	3.1952569	2.16656422	3.177210517
%RSD	0.9813944936	0.5083842529	0.6044537737

Ruggedness: It is the degree of reproducibility of test results acquired by the analysis of the same samples under a various condition, such as various analysts or laboratories. It was determined by measuring the absorbance of Standard (40ppm) at 352nm by different analysts. The Percentage Relative Standard Deviation (%RSD) was determined and results obtained are within the limits as per ICH guidelines.

TABLE 6: RESULTS OF RUGGEDNESS DATA OF SACCHARIN

Concentration	Analyst 1	Analyst 2
40ppm	0.4239	0.3467
40ppm	0.4272	0.3418

40ppm	0.4234	0.3431
40ppm	0.4263	0.3498
40ppm	0.4284	0.3482
40ppm	0.4278	0.3456
Mean	0.426166667	0.345866667
SD	4.306666666	0.003028971
%RSD	1.010559248	0.87576269

Detection Limit (DL): In accordance with ICH guidelines the LOD was calculated using the formula 3.3 x / S for LOD, in which σ is the standard deviation of the response (Y- intercept) and S is the slope of the calibration curve, and the results are obtained.

DL= 3.3 x SD/slope.

= 3.3x0.002075 /0.0182

= 0.362637µg/ml.

Quantification Limit (QL): In accordance with ICH guidelines the LOQ was calculated using the standard formula 10 x / S for LOQ, where is the standard deviation of the response (Y- intercept) and S is the slope of the calibration curve, and the results were obtained.

QL= 10 x SD/slope.

= 10 x 0.002075 /0.0182

= 1.098901µg/ml

Quantification of Saccharin in Extracted Food Products: To an aliquot 5ml containing a working standard 15 µg of saccharin & 1.5ml of Bromine water was added and shakes gently for 2 minutes and few drops of the formic acid were added to remove the excess of bromine. After 5ml of potassium iodide is added to the above solution a yellow-coloured solution is obtained. Then 1ml of CTAB was added and thoroughly shaken, and the solution was made up to 50ml with water and transferred into a 100ml separating funnel. Then the lower layer was extracted at least twice with 3ml of isoamyl alcohol each time. The absorption was determined at a wavelength of 352nm.

TABLE 7: QUANTIFICATION DATA OF SACCHARIN IN EXTRACTED FOOD PRODUCTS

Sample	Absorbance	Amount of saccharin present in food products	FSSAI limits
Candies	1.3780	0.114mg/kg	5mg/kg per day
Tooth paste 1	1.1662	0.416mg/kg	5mg/kg per day
Tooth paste 2	1.1164	0.476mg/kg	5mg/kg per day
Jellies	1.3268	0.119mg/kg	5mg/kg per day
Tooth paste3	1.2432	0.333mg/kg	5mg/kg per day
Candies sample2	1.3436	0.081mg/kg	5mg/kg per day

Method 2: Aspartame: In the process, we have developed and validated by utilizing UV-Visible spectroscopy method. The procedures were approved in accordance with ICH guidelines. The linearity was 10 -175µg/ml for UV Spectroscopic method individually, demonstrating the R^2 of 0.9971. The ultraviolet (UV) spectroscopic technique was found to be linear, accurate, robust, and rugged.

Linearity: Ability to obtain test results which are directly proportional to the concentration of the analyte in the sample.

Procedure: For preparing serial dilutions pipette out 1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5 ml from 100ppm solution and transfer to separate 10mlvolumetric flask and makeup with diluent to yield 10, 25, 50, 75, 100, 125, 150, 170 ppm solution respectively.

TABLE 8: LINEARITY DATA OF ASPARTAME

Concentration	Absorbance
10	0.1432
25	0.2394
50	0.3563
75	0.5056
100	0.6906
125	0.8215
150	1.0199
175	1.1349

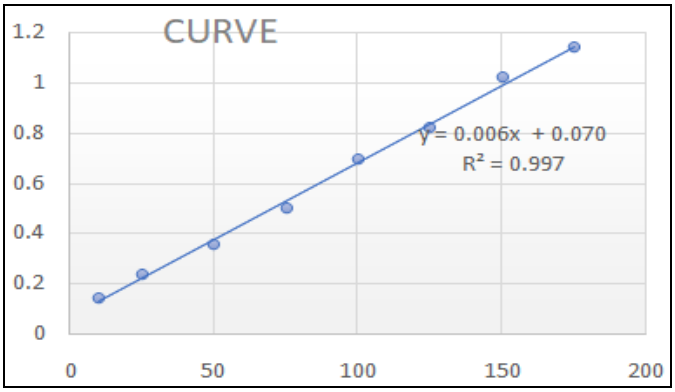

FIG. 6: CALIBRATION CURVE OF ASPARTAME WITH NINHYDRIN

Precision: 6 replicates of 50ppm Standard solutions of Aspartame absorbance was noted at wavelength 570 nm. % RSD was calculated and obtained results are within the ICH Guidelines' limits.

TABLE 9: RESULTS OF PRECISION DATA OF ASPARTAME

Concentration (ppm)	Absorbance
50	0.3563
50	0.3562
50	0.3545
50	0.3553
50	0.3594
50	0.3581
Mean	0.356633333
SD	0.001812917
% RSD	0.508341888

Robustness: It is a measurement of its capacity to remain unaffected by small, but deliberate modifications in the method parameters. It was calculated by measuring the sample absorbance at various wavelengths 569nm, 570nm and 571nm. The Percentage Relative Standard Deviation (%RSD) was calculated and recorded. The %RSD was calculated, and the results are within the limits as per ICH Guidelines.

TABLE 10: RESULTS OF ROBUSTNESS DATA OF ASPARTAME

Concentration (ppm)	Absorbanceat569nm	Absorbance at 570nm	Absorbance at 571nm
50	0.2262	0.3563	0.4262
50	0.2212	0.3562	0.4212
50	0.2231	0.3545	0.4231
50	0.2298	0.3553	0.4298
50	0.2282	0.3594	0.4282
50	0.2246	0.3581	0.4246
Mean	0.2255166667	0.356633333	0.425516666
SD	0.003886273028	0.001812911663	0.003204007901
%RSD	1.723275311	0.5083418913	0.7529688393

Ruggedness: It is the degree of reproducibility of test results acquired by the analysis of the same samples under a different condition, such as various analysts or laboratories. It can be determined by calculating the Standard absorbance (50ppm) at 570nm by different analysts. The %RSD was determined, and the results were according to ICH guidelines, are within the limits.

TABLE 11: RESULTS OF RUGGEDNESS DATA OF ASPARTAME

Concentration (ppm)	Analyst-1	Analyst-2
50	0.3563	0.4262
50	0.3562	0.4212
50	0.3545	0.4231

50	0.3553	0.4298
50	0.3594	0.4282
50	0.3581	0.4246
Mean	0.356633333	0.42551666
SD	0.0018129	3.204007907
%RSD	0.5083418925	0.7529688513

Limit of Detection (LOD): In accordance with ICH guidelines, the LOD was estimated by using the formula 3.3 x / S for LOD, in which σ is the standard deviation of the response (Y- intercept) and S is the slope of the calibration curve, and the results are obtained.

DL= 3.3 x SD/slope.

= 3.3 x 0.0018/0.0061

= 0.980758171µg/ml

Limit of Quantification (LOQ): In accordance with ICH guidelines, the LOQ was calculated by using the standard formula 10 x / S for LOQ, where the standard deviation of the response (Y-intercept) and S is the slope of the calibration curve, and the results were obtained.

DL= 10 x SD/Slope

= 10 x 0.0018/0.0061

= 2.971994457µg/ml

Quantification of Aspartame in Extracted Food Products: 1ml of the sample solution was dissolved in 10ml of ethanol, using 1 ml of the above solution and add 1 ml of acetate buffer mixed with 4ml of Ninhydrin Reagent.

Then the above mixture was then heated in the water bath for 10 minutes. After that, the entire mixture was diluted using 10ml of ethanol, and examine sample solution under the absorbance at 570nm.

TABLE 12: QUANTIFICATION DATA OF ASPARTAME IN EXTRACTED FOOD PRODUCTS

Sample	Absorbance	Amount of aspartame present in food products	FSSAI limits
Ketchup	0.3022	0.5 mg/kg	50mg/kg per day
Soft drink 1	0.1497	1.8 mg/kg	50mg/kg per day
Soft drink 2	0.1394	1.5 mg/kg	50mg/kg per day
Chewing gum	0.1372	2 mg/kg	50mg/kg per day
Candies	0.1578	2.2 mg/kg	50mg/kg per day
Coco cola	0.2661	0.7 mg/kg	50mg/kg per day

CONCLUSION: In conclusion, we have developed a method by using UV-visible spectroscopy for the analysis of quantification is simple, precise, sensitive and accurate method by using less quantities to develop a reproducible result. This approach demonstrates its effectiveness in quantifying and characterizing these artificial sweeteners (Saccharin and Aspartame), offering a cost-effective and rapid technique for quality control in food and beverage industry. Its validation parameters, such as Linearity, Precision, Robustness, Ruggedness, LOD and LOQ affirms the methods suitability for quality control in food industries contributing to ensuring consumer safety. These all the methods developed were validated according to ICH Q2R (2) guidelines. The results of the r^2 value, %RSD of precision, Robustness, Ruggedness of all the methods are within the acceptable limits.

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