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Application of Multibounce Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy and Chemometrics for Determination of Aspartame in Soft Drinks

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Aspartame is a low-calorie sweetener commonly used in soft drinks; however, the maximum usage dose is limited by the U.S. Food and Drug Administration. Fourier transform infrared (FTIR) spectroscopy with attenuated total reflectance sampling accessory and partial least-squares regression (PLS) was used for rapid determination of aspartame in soft drinks. On the basis of spectral characterization, the highest R^2 value, and lowest PRESS value, the spectral region between 1600 and 1900 cm^{-1} was selected for quantitative estimation of aspartame. The potential of FTIR spectroscopy for aspartame quantification was examined and validated by the conventional HPLC method. Using the FTIR method, aspartame contents in four selected carbonated diet soft drinks were found to average from 0.43 to 0.50 mg/mL with prediction errors ranging from 2.4 to 5.7% when compared with HPLC measurements. The developed method also showed a high degree of accuracy because real samples were used for calibration, thus minimizing potential interference errors. The FTIR method developed can be suitably used for routine quality control analysis of aspartame in the beverage-manufacturing sector.

KEYWORDS: Aspartame; carbonated beverages; low-calorie sweetener; HPLC; PLS; FTIR

INTRODUCTION

Low- or reduced-calorie sugar-free foods and beverages are extremely popular worldwide. According to a survey conducted in 2004 by the Calorie Control Council, a trade organization, 180 million adult Americans use these products (*1*). Aspartame (*N*-L-α-aspartyl-L-phenylalanine methyl ester) is a low-calorie sweetener, approximately 180 times sweeter than sugar (*2*). It is currently permitted for food and beverage and/or tabletop sweetener use in more than 100 countries (*3*). As per a report of the American Dietetic Association (ADA) in 2004, the United States leads the world in demand for aspartame, accounting for up to 75% of sales. Although soft drinks account for above 70% of aspartame consumption, this sweetener is added to more than 6000 foods, personal care products, and pharmaceuticals (*4*). The U.S. Food and Drug Administration (USFDA) approved aspartame for use in dry foods in 1981 (*5*) and for use in carbonated beverages in 1983 (*6*). In 1996, the USFDA approved it as a general purpose sweetener for use in all categories of foods and beverages (*7*). The acceptable daily intake of aspartame, which a person can safely consume everyday over a lifetime without risk, is estimated to be 50 mg/kg of body weight (*1, 7*). As per several literature sources, the concentration range of aspartame in commercially available soft drinks generally varies from 200 to 550 ppm (*8, 9*).

However, certain research findings challenging the safety of aspartame indicate that its metabolism products, that is, phenylalanine and methanol, could be toxic (*10*). Phenylalanine is not recommended for phenylketonurics, and elevated doses of phenylalanine can cause changes in behavior (such as depression and insomnia), alteration in vision, and mental retardation, especially in children. An elevated production of methanol can cause acidosis and blindness (*11*).

Considering the safety of aspartame, the restricted content in foods varies among countries. It is, therefore, necessary to develop rapid, simple, and reproducible analytical methodologies under normal laboratory conditions, without the need for sophisticated equipment and specially trained personnel. Thus,

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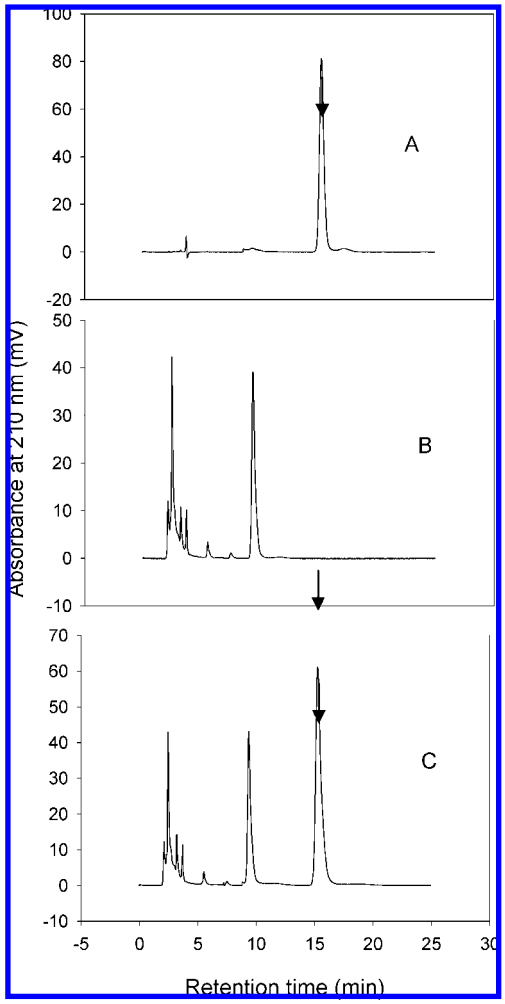

Figure 1. HPLC-DAD chromatograms of aspartame standard (100 mg/L) in water (**A**), extract of aspartame-free brand B soft drink sample (**B**), and extract of aspartame-free brand B soft drink sample fortified with aspartame standard (**C**).

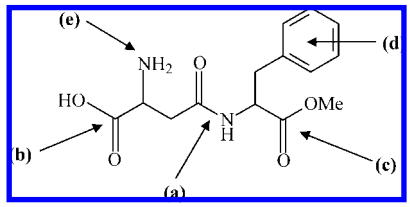

Figure 2. Chemical structure of aspartame: (a) amide group; (b) carboxylic group; (c) ester moiety; (d) monosubstituted benzene ring; (e) primary amino group.

it is very timely and beneficial to address aspartame determination in soft drinks, which cover maximum aspartame usage, in particular.

Several HPLC-based procedures are currently the methods of choice for aspartame determination. Most of these procedures are based on isocratic or gradient reverse-phase (RP) chromatographic separation with acetonitrile, methanol, or isopropanol in phosphate buffer or acetic acid solution as mobile phase and ultraviolet (UV) absorbance detection (*9, 12*). RP ion-pair chromatography (*13*), high-performance anion exchange chromatography (*14, 15*), ion chromatography (*3*), and high-performance capillary electrophoresis (*16*) have also been used for the quantification of aspartame. Several spectrophotometric (*17, 18*) and enzymatic methods (*19*) have also been reported in the literature. Other methods described include electrochemical,

Table 1. Functional Groups and Vibrational Modes of Aspartame FTIR-ATR Spectra

region (cm^{-1})	bond vibration	type of IR absorption[a]	refs
700–1000	aromatic CH stretching	S	*36*
	NH wagging	M	
1000–1300	aromatic CH stretching	M	*31, 34*
	CO stretching	M	
	OCH_3 stretching	M	
	CN stretching	S−M	
	(O═)CO stretching	S	
	CNH bending	S	
1300–1600	aromatic CH stretching	M−W	*34, 35*
	COH bending	M	
	CH stretching/ bending NH_2	M	
	NH_2 scissoring	M	
	OH bending	M	
1600–1900	CO stretching	S	*31, 34*
	aromatic CH stretching	M	
1900–2300	NH bending	M	*35*
	NH stretching	M	
2400–2750	OH stretching	W	*35*
2800–3200	OH stretching	S	*35*
	NH stretching	M	
	aromatic CH stretching	M	

[a] M, medium; S, strong; W, weak; M−W, medium to weak; S−M, strong to medium.

amperometric detection (*20*) and sequential flow injection coupled with enzymic detection (*21, 22*). Most of these procedures are time-consuming or high costs involved; some require intensive sample pretreatment, and many rarely have the selectivity required for commercial samples. Almost all of the methods use reagents that require special precautions in handling and storage.

Fourier transform infrared spectroscopy (FTIR) coupled with attenuated total reflectance (ATR) is a fast and nondestructive alternative to conventional methods. Previous works have shown that FTIR spectroscopy has been useful in the quantification of several important analytes in food samples such as sugars (*23–25*), caffeine (*26*), and phytates (*27*). FTIR spectrometers provide greater energy throughput by using interferometers, scan quickly, and have the capability of coadding data so that the spectra can be obtained rapidly with acceptable signal-to-noise (S/N) ratios (*28*). Multivariate methods such as partial least-squares regression (PLS) are useful in developing prediction models and extracting subtle information from digitized FTIR spectra that might contain overlapping peaks, interference bands due to water or carbon dioxide, and instrumental artifacts due to measurement conditions (*29, 30*).

Increasing requirements and cost pressures nowadays force the government and commercial food-testing laboratories as well as production houses to replace traditional reference methods with faster, environmentally friendly, and more economical ones. Armenta et al. (*31*) proposed a procedure for the determination of aspartame in tabletop sweeteners; however, the multivariate analysis was not applied. As far as can be determined from the accessible literature, FTIR in conjunction with chemometrics has not been investigated for aspartame quantification. In this study, FTIR in combination with PLS regression has been evaluated as an alternative method for aspartame determination. The specific objectives of the present work were to (i) investigate

Table 2. Calibration and Validation Statistics for the Model Developed for Brand A and B Samples Using FTIR-ATR and Chemometrics[a]

	brand A						brand B					
			calibration		validation				calibration		validation	
wave no.	factors	RMP	R^2	SEC	R^2	SEP	factors	RMP	R^2	SEC	R^2	SEP
700–1000	2	0.589	0.914	6.262	0.904	7.566	1	0.772	0.698	10.911	0.751	10.333
1000–1300	7	0.215	0.986	2.278	0.977	3.759	5	0.328	0.972	3.785	0.966	4.437
1300–1600	5	0.303	0.959	4.573	0.935	5.339	4	0.279	0.969	3.892	0.939	5.248
1600–1900	7	0.201	0.991	2.023	0.980	3.177	7	0.155	0.995	0.955	0.997	2.007
1900–2300	9	0.249	0.977	2.581	0.898	6.702	8	0.264	0.984	2.185	0.962	2.135
2400–2750	4	0.385	0.956	4.655	0.959	4.629	4	0.420	0.969	3.931	0.951	5.223
2600–3000	5	0.393	0.942	5.391	0.810	9.235	6	0.314	0.967	4.171	0.976	3.473
2800–3200	4	0.396	0.955	4.732	0.941	5.139	2	0.568	0.798	9.314	0.879	7.202

[a] Factors, optimum number of factors extracted; RMP, root mean press value; R^2, coefficient of correlation; SEC, standard error of calibration; SEP, standard error of prediction.

Table 3. Calibration and Validation Statistics for the Model Developed for Brand C and D Samples Using FTIR-ATR and Chemometrics[a]

	brand C						brand D					
			calibration		validation				calibration		validation	
wave no.	factors	RMP	R^2	SEC	R^2	SEP	factors	RMP	R^2	SEC	R^2	SEP
700–1000	2	0.468	0.911	6.366	0.923	5.791	2	0.564	0.909	6.256	0.901	7.421
1000–1300	6	0.260	0.970	2.288	0.963	2.968	5	0.283	0.971	2.314	0.964	3.156
1300–1600	8	0.289	0.905	2.665	0.849	4.747	4	0.272	0.968	3.514	0.951	4.691
1600–1900	8	0.193	0.993	2.038	0.986	2.744	7	0.182	0.993	2.016	0.998	2.262
1900–2300	6	0.231	0.942	2.238	0.970	3.836	8	0.243	0.986	2.541	0.903	6.142
2400–2750	2	0.461	0.878	7.389	0.887	7.035	2	0.397	0.969	4.145	0.956	4.713
2600–3000	8	0.222	0.974	2.233	0.970	2.879	5	0.324	0.970	4.342	0.973	3.89
2800–3200	2	0.553	0.849	8.140	0.764	9.679	3	0.514	0.881	7.682	0.792	7.142

[a] Factors, optimum number of factors extracted; RMP, root mean press value; R^2, coefficient of correlation; SEC, standard error of calibration; SEP, standard error of prediction.

Table 4. Predicted Concentration of Aspartame in Validation Set of Different Soft Drink Samples Using PLS Using the Region between 1600 and 1900 cm^{-1}

	predicted aspartame concn (mg %)			
actual aspartame concn (mg %)	brand A	brand B	brand C	brand D
3.000	4.898	3.500	4.352	4.788
7.000	10.325	9.355	4.222	8.645
11.000	11.728	13.041	15.181	15.181
15.000	18.046	15.737	14.853	17.483
19.000	21.890	21.146	20.022	21.320
25.000	29.395	24.683	25.428	25.893
31.000	27.686	32.764	26.186	33.856
37.000	30.995	37.466	37.084	38.145
43.000	42.322	46.803	46.900	45.362
49.000	50.141	51.704	51.934	49.620

aspartame content in commercial soft drinks using FTIR-ATR and chemometrics and (ii) validate the results using a conventional HPLC method.

MATERIALS AND METHODS

Samples. Four diet soft drinks, brands A, B, C, and D, containing aspartame as sweetener and corresponding regular soda products containing sugar were obtained from a local market. Standard aspartame was obtained from Sigma-Aldrich Co. (St. Louis, MO).

Preparation of Calibration and Validation Data Sets. The regular (aspartame-free) soft drinks were used to prepare samples with different concentrations of aspartame expressed as percent by weight of soft drinks. Four calibration sets (one each for brands A−D) were prepared. For each set, the regular soft drink samples were separated into two parts, one with aspartame added and the other without aspartame. These were then blended in appropriate proportions to obtain 51 samples for each soft drink with aspartame concentration ranging from 0 to 50 mg % (0−50 mg/100 mL). Of these, 41 were used for calibration, and the rest were used for external validation. The total volume of each sample prepared was 20 mL.

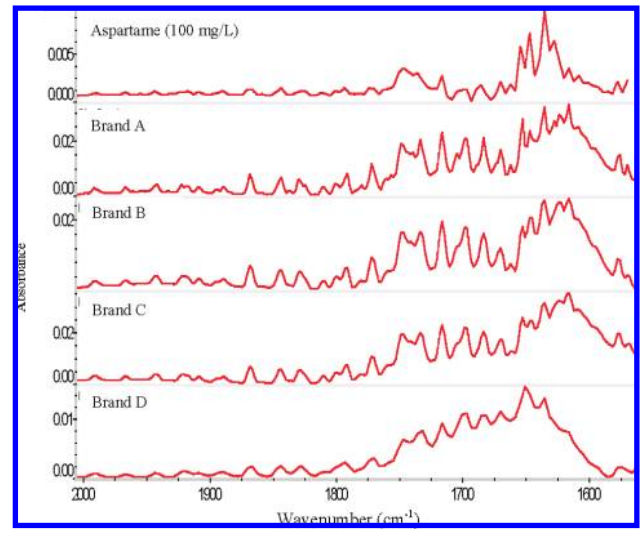

Figure 3. Stacked FTIR spectra of aspartame standard (100 mg/L) in water and brand A−D soft drink samples in the range between 1600 and 2000 cm^{-1}.

FTIR-ATR Instrumentation and Spectral Collection. A Nicolet 6700 spectrometer (Thermo Electron Corp., Madison, WI) equipped with a deuterated triglycine sulfate (DTGS) detector and Smart Attenuated Total Reflectance kit (ARK) (Thermo Electron Corp.) was used for the measurement. The Smart ARK is an advanced multibounce horizontal attenuated reflectance (HATR) accessory, producing 12 reflections with a penetration depth (infrared beam) of 2.0 μm. The accessory comprised a ZnSe crystal for sample containment with an aperture angle of 45° and refractive index of 2.4 at 1000 cm^{-1}.

The soft drink samples were degassed and tempered to room temperature (22 °C) before spectral collection. Single-beam spectra (4000–400 cm^{-1}) of the samples were obtained at a resolution of 8 cm^{-1} and a total of 64 coadded scans. The ATR crystal was carefully

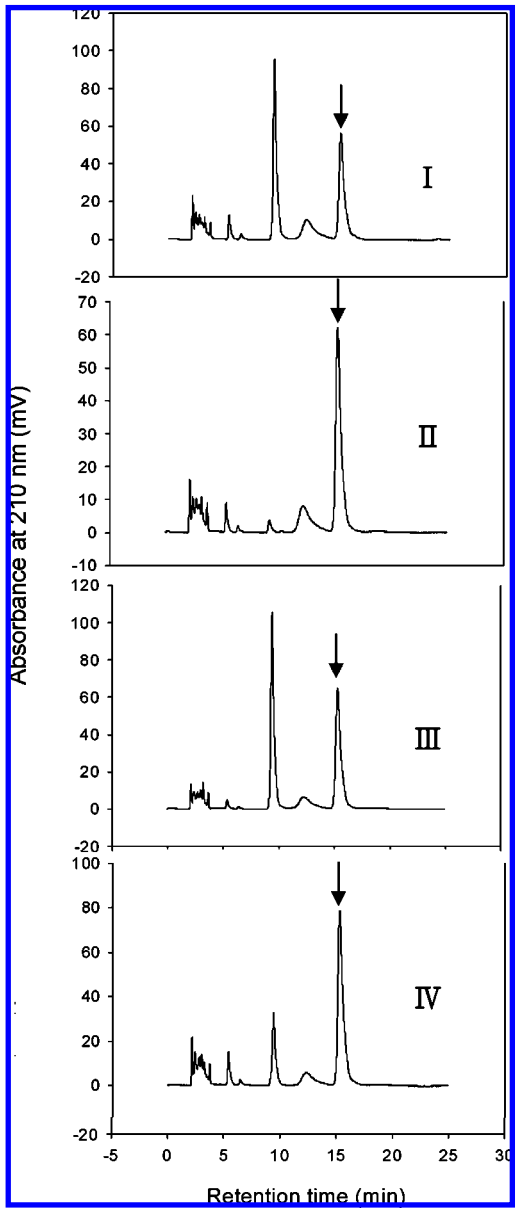

Figure 4. Typical HPLC-DAD chromatograms of brand A extract (**I**), brand B extract (**II**), brand C extract (**III**), and brand D extract (**IV**).

Table 5. Comparison between Predicted Contents of Aspartame in Commercial Soft Drink Samples Using FTIR-ATR and HPLC

sample	predicted concn[a] by FTIR-ATR (mg %)	determination[a] by HPLC (mg %)	prediction error (%)
brand A	48.25	45.64	5.72
brand B	49.67	48.51	2.39
brand C	42.96	40.83	5.22
brand D	47.88	46.66	2.61

[a] Values are averages of triplicates.

cleaned with Milli-Q water and 75% ethanol and dried using compressed air to eliminate the presence of any residues between measurements. Each experiment was replicated three times, and the spectra were averaged before they were subjected to multivariate analysis.

Chemometrics. SAS 9.1 (SAS Institute Inc., Cary, NC) was used for PLS analysis on the spectral data obtained from OMNIC software (Thermo Electron Corp.). To model the system without overfitting the concentration data, a cross-validation method based on split sample validation was used, wherein successive groups of widely separated observations were held out as the test set. R^2 values, predicted residual error sum of squares (PRESS) value, number of factors in the model, standard error of calibration (SEC), and standard error of prediction (SEP) values of the respective calibration and validation data sets were used to test the predictability of the models. The number of factors optimized to minimize the PRESS and maximize the R^2 value was selected (*32*). The SEC and SEP were calculated as shown in eqs 1 and 2.

$$\text{(calibration data) SEC} = \sqrt{\frac{\sum_{i=1}^{n}(\text{actual} - \text{predicted})^2}{n-f-1}}$$

$$\text{(valiation data) SEP} = \sqrt{\frac{\sum_{i=1}^{n}(\text{actual} - \text{predicted})^2}{n}}$$

"Actual" refers to the concentration of aspartame in the sample, and "predicted" refers to the concentration computed by PLS of the sample spectra; n is the number of samples in the calibration set, and f refers to the number of factors in the calibration model. The undesirable variations in the spectra were removed using the mean centering and autobaseline methods.

Estimation of Aspartame by HPLC Method. Concentrations of aspartame in the diet soft drink samples were estimated using the RP HPLC method suggested by Prodolliet and Bruelhart (*12*) with slight modifications. The LC instrument was an Agilent 1100 (Agilent, Palo Alto, CA) equipped with a diode array detector (DAD), a binary pump, an ALS autosampler, and a thermostated column compartment held at 20 °C. A 1 mg/mL aqueous stock solution of aspartame was prepared by dissolving 100 mg of aspartame in 100 mL of HPLC grade water. Different working standards of 20, 40, 60, 80, and 100 μg/mL were prepared from stock solution. The soft drink samples were diluted 5 times with HPLC grade water, filtered through 0.22 μm nylon membrane filters, and passed through C 18 Sep-Pak Classic cartridges (Waters Co., Milford, MA) before injection into the HPLC system. Separation of aspartame was carried out on a C18 column (Inertsil ODS-3V, 5 μm, 4.6 × 250 mm, GL Sciences Inc.). The solvent system was isocratic with 90% of 0.0125 M KH_2PO_4 (pH 3.5, adjusted using 5% phosphoric acid) and 10% of acetonitrile, and the flow rate was adjusted to 0.8 mL/ min. Twenty microliters of the sample was injected, and detection was done at 210 nm absorbance. Each sample and standard was injected three times. For calculating recovery a regular soft drink sample was spiked at two levels (50 and 100 mg/L) with aspartame, and sample preparation was carried out as mentioned above for diet soft drinks.

The calibration equation obtained in the aforementioned conditions was $A = 1117.7 \times (C_{AS}) - 53.14$, with an R^2 value of 0.996, where A and C_{AS} refer to the area value and concentration of aspartame in milligrams, respectively. As depicted in the **Figure 1**, a representative recovery test was performed in aspartame-free soda product, such as regular cola of brand B, at two fortification levels (50 and 100 mg/L). The average recoveries of aspartame from the brand B sample were in the range of 86.9−90.5%.

RESULTS AND DISCUSSION

Figure 2 shows the chemical structure of aspartame. The different functional groups and their respective bond vibrations are together responsible for the IR spectra of aspartame. As noted in **Figure 2**, the key functional groups leading to characteristic IR spectra of aspartame include the (a) amide group, (b) carboxylic group, (c) ester moiety, (d) monosubstituted benzene ring, and (e) primary amino group. CO and CN stretching in the amide portion could be responsible for exhibiting strong IR absorption in the regions around 1650 and 1550 cm^{-1}, respectively (*33*). The ester group characterized by carbonyl bonds could be accounted for by strong IR absorption bands in the region around 1715–1750 cm^{-1} (*34*). In addition, there is a strong IR band cluster in the region of 1150–1300

cm^{-1}, which might be attributed to (O═C)—O stretching. This band constitutes a high-frequency single-bond stretch involving CC, CO, and OC bonds. The IR bands around 3000 cm^{-1} may be assigned to OH stretching vibration in the carboxyl moiety (*31, 35*). Similarly, strong wagging of aromatic CH belonging to the monosubstituted benzene might have led to the occurrence of bands around 700–800 cm^{-1} region. In addition, the bands around 1050 and 1600 cm^{-1} could be assigned to CN stretching and NH_2 scissoring, respectively (*35*).

When latent variable methods such as PLS are employed to build calibration models, the absorbing regions of the spectra dictate the number of variables used as input in the multivariate statistical model. In the present study, PLS was employed to develop multivariate calibration models from a set of 41 samples of each soft drink for predicting aspartame. The spectra obtained were divided into seven regions (700–1000, 1000–1300, 1300–1600, 1600–1900, 1900–2300, 2400–2750, and 2800–3200 cm^{-1}) to build calibration and validation models for aspartame determination on the basis of different bond vibrations in the spectra of aspartame. **Table 1** summarizes the important bond vibrations in these regions as related to aspartame structure.

The calibration models developed for the four soft drink samples were validated using an external set of 10 samples for each soft drink. **Tables 2** and **3** present the PLS analysis of the different spectral regions obtained from the calibration and validation sets prepared using soft drink samples spiked with aspartame. The tables present R^2, root mean PRESS, SEC, and SEP values for all seven regions selected. It could be inferred that PLS analysis in the spectral region of 1600–1900 cm^{-1} resulted in the highest R^2 value, which is an indicator of degree of fit, as well as the lowest root mean PRESS value, which corresponds to the average error in the analysis. Moreover, the SEC and SEP values were also found to be the lowest in the same region. Additionally, the spectral region within 1600–1900 cm^{-1} constitutes important absorbance bands related to stretching vibrations of carbonyl bonds in the aspartame molecule. Thus, selection of this region for the analysis of aspartame in real samples of diet soft drinks is fully addressable. As per **Tables 2** and **3**, seven factors were extracted for brand A, B, and D soft drink samples, whereas eight was the optimum number of factors extracted for brand C in the selected spectral region from 1600 to 1900 cm^{-1}.

Table 4 shows the results of PLS predictions compared with the actual values for 10 validation samples of each soft drink obtained from the selected spectral region from 1600 to 1900 cm^{-1}. The predicted concentrations of aspartame in the validation samples were in good agreement with the actual values for all four soft drink samples with a minimum correlation coefficient of 0.980.

The FTIR procedure and calibration models developed for aspartame estimation were tested on real diet soft drink samples of four different commercial brands (brands A−D). The ATR-FTIR spectra of the four diet soft drinks and pure aspartame in water (100 mg/ L) in the 1600–1900 cm^{-1} spectral region are shown in **Figure 3**. The corresponding chromatograms for the soft drink samples obtained from HPLC analysis are presented in **Figure 4**.

Table 5 shows the concentration of aspartame in four diet soft drink samples as predicted by FTIR-PLS in comparison with those obtained from HPLC. The values quantified using HPLC were treated as true values, and percent error in prediction was calculated. There was a good agreement between FTIR predictions and HPLC results with prediction errors of 5.7, 2.4, 5.2, and 2.6% for brand A, B, C, and D soft drink samples, respectively. The results obtained from FTIR were slightly higher than those obtained by the standard HPLC method; however, the absence of wet chemicals and rapid analysis make the FTIR an effective tool for aspartame determination.

Moreover, in this study the regular soft drink counterparts were spiked with standard aspartame and used for the calibration set, which gave a better correlation because the contribution of components other than aspartame was also incorporated into the calibration. The developed method is very rapid; however, the average error in prediction is high at low levels of analyte, which could possibly be attributed to noise produced due to change in conditions and less sensitivity at low aspartame concentration. The appropriate range could be 25–50 mg %.

The FTIR procedure validated using HPLC results highlights that aspartame could be estimated in the commercial soft drink samples in less than 5 min without any physical/chemical sample manipulations. The developed method shows excellent promise as a rapid, effective, and environmentally friendly tool for inline and online analysis of aspartame in soft drinks.

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