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Development, Optimization and Validation of a RP-HPLC-UV Method for the Determination of Benzoic Acid in Selected Food Products

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ABSTRACT
A reversed phase high-performance liquid chromatography method with ultraviolet detection (RP-HPLC-UV) had been established and evaluated for determining benzoic acid in selected commercial food products. the C-18 analytical column (25.0cm × 4.6 mm, 5 µm) and the isocratic methanol–water mobile phase (40: 60 , v/v) using glacial acetic acid adjusted to pH 3.5 were used for chromatographic separation. The detection was done at 228 nm, the temperature of column was 25 °C, and the rate of flow was 1.0 mL per min. Benzoic acid showed a retention duration of about 4.24 minutes under the chosen circumstances. With a coefficient of determination of 0.9997, the technique demonstrated a linear calibration response over 1.0–50.0 µg/mL. The detection limit and quantification limit were reported to be 1.04 and 3.16 µg/mL, respectively. With relative standard deviations less than 1.4%, recovery values for the examined beverage matrices varied from 98.88% to 101.55%. Benzoic acid values in commercial food samples were 120.5 mg/L in orange juice, 145.0 mg/L in cola drink, and 45.2 mg/kg in tomato paste. Under analytical conditions, benzoic acid was not found in apple nectar These results show that the examined matrices have good analytical performance. Before the approach may be deemed adequately validated for routine food-safety monitoring, additional clarification about extraction processes, selectivity, validation calculations, and applicable regulatory limits is required.

Keywords: Benzoic acid, Food preservatives, RP-HPLC, Food safety, Method validation

INTRODUCTION

The fundamental principle of the modern food industry lies in modern food-preservation techniques, which help to prevent microbial decay and prolong the food's shelf lifetime.. Benzoic acid ($C_7H_6O_2$) and its salts, such as potassium benzoate and sodium benzoate, are using to preserve acidic foods and drinks and extend their shelf life thanks to their antimicrobial activity. The effectiveness of these preservatives is linked to benzoic acid's capability to inhibit the growth of a broad class of microorganisms, such as yeasts and fungi. However, as the consumption of large quantities of preservatives, particularly benzoic acid, may raise concerns regarding toxicity and public health among individuals who consume such foods, their levels in processed foods must be strictly controlled. For these reasons, international food safety authorities supervise the processes of using food preservatives, with levels of use regulated by food categories and applicable legislation(Abedi, A., & Khezeli, T. 2024; So, J. S., etal, 2023; Werner, J., & Mysiak, D. ,2024; Alabyadh, M. T. ,2023).

Types of analytical techniques have developed to determine benzoic acid and other food preservatives, as such liquid chromatography with high performance and gas chromatographic (Awagu & Okoroafor, 2025), spectrophotometric methods and capillary electrophoresis (Zhang, Y. et al. ,2025; Wang, L., et al. (2024), Among these techniques, Benzoic acid in food products has been extensively analyzed using reversed phase high performance liquid chromatography coupled with the UV detector, owing to its good separation efficiency, accuracy, sensitivity and ease of sample preparation. Furthermore, when appropriate chromatographic conditions are used this technique can simultaneously determine benzoic acid and other preservatives (So, J. S., etal, 2023; Nomura, C., etal, 2024).

Many recent analytical studies have focused on improving extraction efficiency, reducing solvent consumption and analysis time, and developing more reliable and environmentally friendly procedures for sample preparation (Liao, Y., etal, 2026). At the same time, a fundamental requirement for demonstrating the Relevance of a technique for the quantitative analysis is the appropriate validation of analytical methods, which provides important evidence regarding performance of the analytical procedure, as well as parameters that include linearity, sensitivity, precision, accuracy, detection limit, and quantification limit (de Oliveira Arias, et al, 2026; Das, S., etal, 2024; Huy, D. H., etal, 2026). In this setting, the study aims to develop and optimize the extraction and identification of benzoic acid, to validate the reversed phase HPLC approaches, and to quantify it in selected commercial food products, including processed tomato paste, carbonated drinks, and canned fruit juices. The suggested approach was evaluated of linearity, sensitization, precision, accuracy, and replication and subsequently utilized to investigate of marketing food samples from local markets. The study also provides a statistical evaluation of the analytical performance and the suitability of the techniques of the regular analysis of benzoic acid in foods products.

MATERIALS AND METHODS

Reagents, Chemicals and Instrumentation

The benzoic acid reference standard was purchased from Sigma Aldrich (USA), with a reported purity of 99.98%. Methanol for HPLC analysis has a purity of ≥ 99.9% was provided from Merck (Germany), while high-purity water has a purity of 99.98%, glacial acetic acid has a purity of ≥99.7%

to 99.8% assay, and other materials were obtained from SDI, Samarra, Iraq. The mass of the reference material was corrected for its reported purity. Accordingly, 25.0050 mg of the reference standard was weighed, placed in mobile phase, and diluted to the final volume of 250 mL to obtain a stock solution equivalent to 25.00 mg of pure benzoic acid, corresponding to 100.00 µg/mL of concentration.

The HPLC components was equipped to C18 column a Thermo Scientific® BDS Hypersil (250 × 4.6 mm, 5 µm), LC-20AD pump, DGU-20A degasser, SIL-20AC autosampler, CTO-20AC column oven, and SPD-20A detector. These components were connected to a computer running Win7 Software. The UV Probe- 2.42 software was utilized with the Shimadzu UV-1800 UV–Vis dual beam spectrophotometer to determine λ_{max} for benzoic acid when dissolves in the optimal mobile phase ratio. A HANNA® HI 8314 pH meter (Romania) was using for a pH measurement and adjustment. An Auto Edu ultrasonic device (China) was used for ultrasonic treatment and degassing, as applicable**.**

Chromatographic Conditions

Benzoic acid was separated chromatographically using a Hypersil C-18 reversed phased analytical column (25cm × 4.6 mm inner diameter, particles size 5 µm) and mobile phase of MeOH and H_2O in the 40 : 60 (v/v) ratio. Glacial acetic acid was utilized to bring a pH of the mobile phase down to 3.5 after mix methanol and water. The mobile phase was then filtering via 0.45 µm filter membrane from Mille pore and degassing using Auto Edu Ultrasonic (China). Before sample analysis, The phase of mobility had been balanced in the column until a stable baseline and consistent retention time were obtained. benzoic acid was came out about 4.24 minutes then chromatography running time was 10 min.

The mobile phase was pumped at speed rate in 1.0 ml/min, Validation was done at λ_{max} of 228 nm, The column's temperature was 25 °C, and Evaluation was done under a series of separation conditions using injected volume of 20.0 µL, which were used to determine benzoic acid in the food samples under study. The different food samples were analyzed using the benzoic acid retention time.

The assigned chromatographic settings were used to analyze the food samples, and benzoic acid was estimated by compare its retention duration with the reference standard. System suitability was assessed using specify the parameters evaluated, like retention time repeatability, peaks areas precision, peaks symmetry or theoretical plate number.

The stock solution was serially dilutes with the mobile phase to prepare stock solutions to HPLC analysis. The range of concentrations from 1.00 to 50.00 µg/mL was used to construct calibration standards. Each calibration concentration was prepared using the precise aliquot and final dilution volumes. The calibration response and repeatability were assessed using the data obtained from the five injections of each standard solution.

Samples of commercially available food products, including processed tomato paste, beverages, and bottled fruit juices, were collected from local markets in Samarra, Iraq. Ten samples in all, representing one batch and six various brands, were studied. Before analysis, Products remained in suitable conditions at room temperature. After being collected, all samples were examined within two weeks.

Preparation of Benzoic Acid Stock Solution

A benzoic acid stock solution (100 µg/mL) was established by weighed a 25.0050 mg of benzoic acid (purity 99.98%) dissolving it in mobile phase, and making the solution to the final volume of 250 mL. A weighed mass was corrected for the reference-standard purity to obtain an amount equivalence to 25.00 mg from pure benzoic acid. The stock solution was serially diluted with the mobile phase to prepare a required working solutions for HPLC evaluations.

Samples Collection

commercially Samples available food products, including processed tomato paste, beverages, and bottled fruit juices, were collected from local markets in Samarra, Iraq. during March 2026. A total of 10 samples representing 6 different brands and one batch were investigated. in suitable conditions at room temperature under appropriate conditions until analysis. All samples were analyzed within two weeks of collection.

Liquid Food Samples

Before analysis, the fruit juice and beverage samples were thoroughly homogenized. Gases were removed from carbonated samples by ultrasonic treatment for 15 min to minimize their potential effects during chromatographic analysis. The same ratio of mobile phase above was used to dilute an aliquot of ten mL of any sample to final volume in a 25 mL volumetric flask. The resulting dilution factor was 2.5, There were no more extraction procedures carried out. When determining the benzoic acid concentrations in the original samples, represented as mg/L, the dilution factor was taken into consideration. The supplied samples were filtrate through a 0.45 µm membrane filter and put straight into HPLC vials prior to analysis under the ideal chromatographic conditions. When determining the benzoic acid concentrations in the initial samples, which were stated in mg/L, the dilution factor was considered.

Tomato Paste Samples

Before extraction, the tomato paste samples were homogenized. A 5.0000 g portion of sample was weighing into a suitable centrifuge tubes, and 50 mL of a methanol: water mixture (40:60, v/v) was added as extraction solvent. The mixture was thoroughly blended using a vortex mixer and sonicated for 15 min to facilitate the transfer of benzoic acid into the extraction solvent. To separate the liquid extract from the solid residue, the mixture was rounded for fifteen minutes at about 4500 rpm. The extraction process was carried out once, and the supernatant was gathered. Without further extraction, the obtained supernatant was utilized for further investigation. 35 milliliters was the final extract volume. Before analysis, the resultant extract was filtrate through a 0.45 µm membrane filter into an HPLC vial. The measured extract concentration, the final extract volume, the initial sample quantity, and all dilution factors were utilized to calculate the benzoic acid content in tomato paste in mg/kg.

Method Selectivity

The chromatogram of the benzoic acid stock solution and the examined food samples were contrasted in order to evaluate the selectivity of The proposed methodology. Benzoic acid's retention period (around 4.24 minutes) and lacks of notable interferences peaks for analyst's retention time were used to determine its existence.

RESULTS AND DISCUSSION

Optimization and Development of Chromatographic Conditions

In order to achieve a separation as close as possible to the ideal, the chromatographic conditions were systematically calibrated to yield a symmetrical peak shape, a reproducible retention time and good sensitivity for evaluated of benzoic

acid in selected foodstuffs. The optimization process involved evaluating the mobile phase content, injected volume and chromatographic column; the conditions were then used to validate the method and analyzed commercial food samples Figure1 show spectrum of benzoic acid.

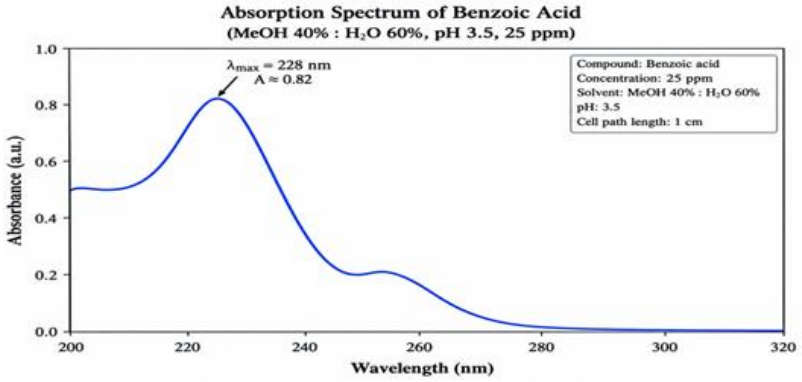


Figure 1: spectrum of benzoic acid

Selection of the Mobile Phase

Five distinct methanol–water ratios (30:70, 35:65, 40:60, 45:55, and 50:50, v/v) were used to examine the impact of mobile-phase composition on the chromatographic separation of benzoic acid. Table 1 summarizes the related chromatographic results, which include retention duration, peak area, peak height, and tailing factor. Figure 2 shows representative chromatograms produced with various mobile-phase compositions.

As showed in the Table 1 and Figure 2, increasing the methanol proportion resulted in a progressive decrease in the retention time, from 6.14 min at 30:70 to 3.25 min at 50:50 (v/v). However, as differences in peak symmetry were also noted, a shorter retention period did not always imply improved chromatographic efficiency. A virtually symmetrical peak was indicated by the tailing factor's decline from 1.45 at 30:70 to 1.28 at 35:65 and its lowest value of 1.08 at 40:60 (v/v). The tailing factor increased to 1.25 and 1.48 at 45:55 and 50:50, respectively, at greater methanol concentrations, indicating a decline in peak symmetry.

With a retention period of 4.24 minutes, the highest peak height (412 mV), and the lowest tailing factor, the 40:60 mixture offered a superior overall chromatographic profile, despite the 50:50 composition producing the highest peak area (519,213). Because it offers a good compromise between retention time, detector response, and peak symmetry, the ratio of methanol–water 40:60 (v/v) was chosen as the ideal mobile-phase composition for ensuing analyzes.

Table 1: Optimization of Methanol–Water Mobile Phase Components

Methanol: H_2O (v/v)	Rt (min)	Peaks area	m.v	T.f
30:70	6.14	442,851	358	1.45
35:65	5.18	472,731	383	1.28
40:60	4.24	493,891	412	1.08
45:55	3.57	512,183	385	1.25
50:50	3.25	519,213	371	1.48

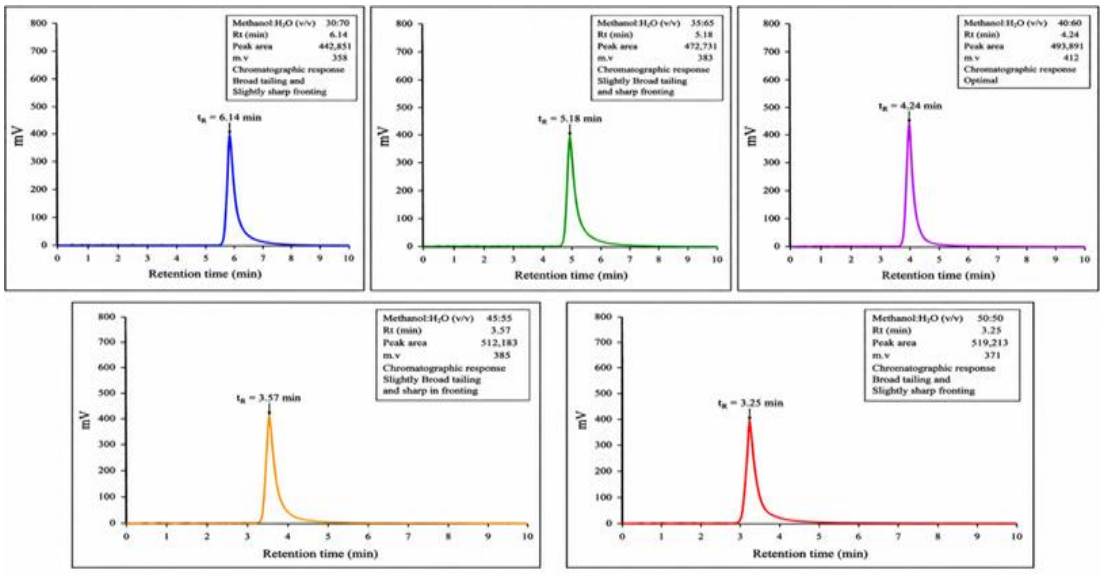


Figure 2: Representative HPLC chromatograms of benzoic acid obtained using different methanol–water mobile-phase compositions

Selection of the Best Chromatographic Column

A range columns was selected on the basic of retention time, selectivity and peaks shape for benzoic acid in foodstuffs, utilizing reversed phase high performances liquid chromatography. Consequently, several columns were evaluated, including C8, Hypersil C18, phenyl and a shorter C18 column; Considering the outcomes, the C18 was selected for the analysis as it produced a Clearly defined peak with good symmetry, a stable retention time and an adequate response

The excellent performance of Hypersil C18 column can be ascribed to its nonpolar stationary phase, which offers suitable hydrophobic interactions with the relatively non-polar form of benzoic acid under the chosen acidic mobile phase reasons. In addition, the column length of 250 mm allowed for good Analyte-stationary phase interaction, helping to achieve good separation and reproducibility as shown in Table 2.

Table 2: Selection of the Best Column

Column	Rt. (min)	Peak Shape	Overall Performance
C8	3.82	Acceptable	Good
Hypersil® C18 (25.0cm × 4.6 mm, 5 µm)	4.24	Symmetrical	Optimal
Phenyl	4.56	Acceptable	Good
Short C18	2.94	Less symmetrical	Moderate

Selection of Injection Volume

In order to select the optimal injected volumes of 10, 15, 20 and 25 µl were tested under the previously optimized chromatographic reasons. The tests showed that gradually raising the injection's volume between 10 to 20 µl led to an increase in the detector's response without any major change in the peak shape. At 30 µl, a slight broadening for the peaks were recorded., suggesting possibility of samples overload, which is consistent of the capacity of the separation column. Therefore, 20 µl was chosen as the optimal injection volume as it provided good reproducibility and sufficient chromatographic response for the detector, with a symmetrical peak as shown in Table 3 and Figure 3.

Table 3: Selection of Injection Volume

Injection volume (µL)	Peak response	Peak shape	Assessment
10	Low	Good	Insufficient response
15	Moderate	Good	Acceptable
20	High	Symmetrical	Optimal
25	Very high	Slight broadening	Not preferred

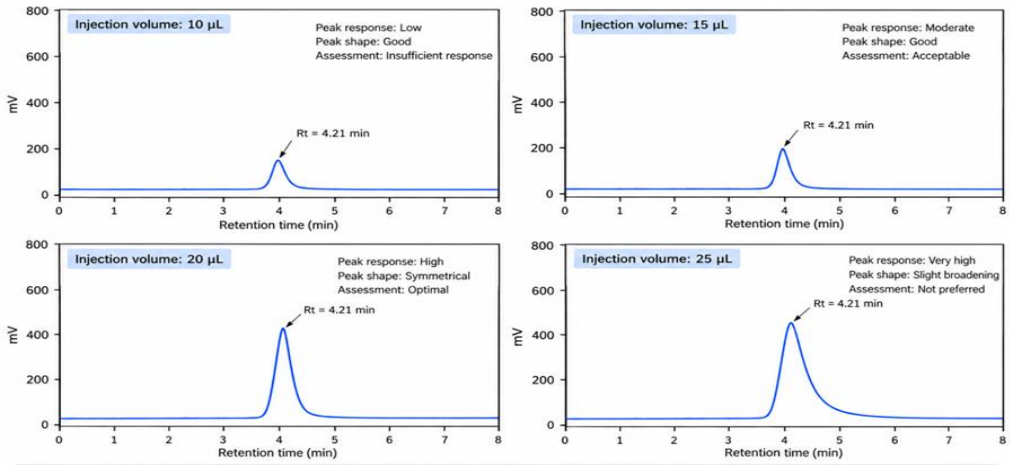


Figure 3: Shapes the peaks in different Injection Volume

Selection the Rates of Flow for Mobile Phase

In order to select the Rates of Flow for Mobile phase, the following the Rates of Flow were tested: 0.8, 1.0, 1.2 and 1.5 mL/min. Flow rate of 0.6 and 0.8 mL/min led to a rise in analysis duration, whilst a 1.00 mL/min flow rate of reduced analysis time and produced an excellent peak with good reproducibility. whereas the higher flow rates of 1.2 and 1.5 mL/min reduced retention time, resulting in tailing for peaks and a reduced response. For these reasons, a 1.0 mL/min was selected, as shown in Table 4 and Figure 4.

Table 4: Selection Rates of Flow of Mobile Phase

F. R. (mL/min)	Rt	Peak shape	Assessment
0.6	Long	Sharp and symmetrical	Long analysis time
0.8	Relatively long	Sharp and symmetrical	Acceptable, but slow
1.0	Moderate	Well-defined and symmetrical	Optimal
1.2	Short	Slightly broadened	Reduced resolution
1.5	Very short	Slight distortion	Not preferred

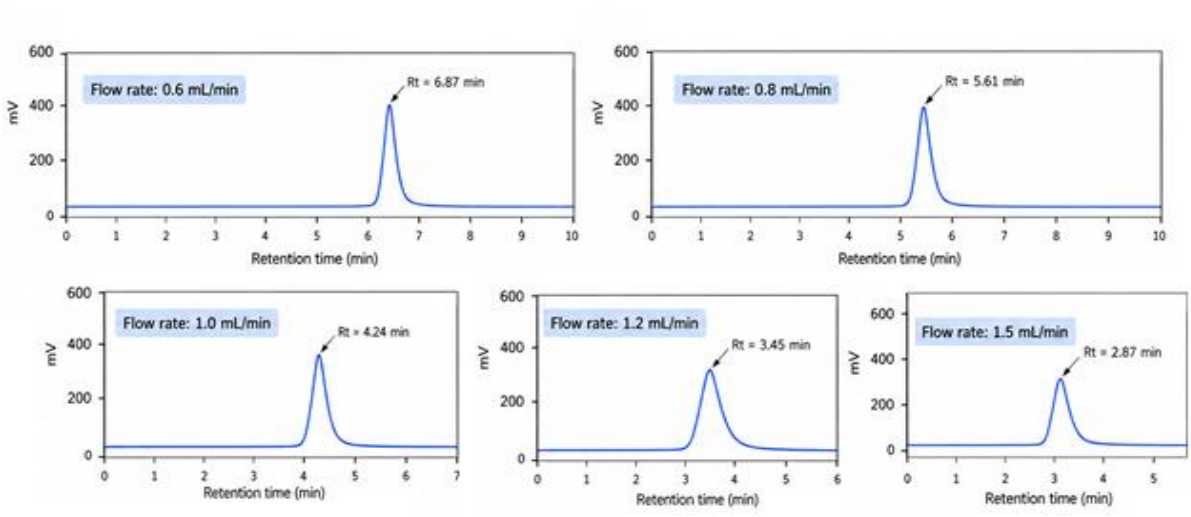


Figure.4: show effect of different flow rates

Optimized Chromatographic Conditions

A methanol–water mobile phase (40:60, v/v) that had been modified to pH 3.5 by used glacial acetic acid to carry out the separation under isocratic conditions. column's temperature was fixed to 25.0 °C, the flow rates was kept at 1.0 mL/min, and a 20 µL injection volume was used for UV detector at 228 nm. The mobile phase was used to balance the column till a stable the baseline is achieved show in Table5. The chromatographic run took ten minutes in total. Five consecutive injections for solutions of benzoic acid at single concentration were used to enhanced the appropriateness system.

Table 5: The Selected Chromatographic Conditions are Summarized Below

Parameter	Reported Condition
Analytical column	BDS, Hypersil C-18
Column dimensions	25.0 cm × 4.6 mm
Particles size	5.0 µm
Mobile-phase	Methanol–water (40:60, v/v)
Mobile-phase pH	3.5
Elution mode	Isocratic
Flow rate	1.0 mL/min
Column temperature	25 °C
Detection wavelength	228 nm
Injection volume	20µL

Analytical Methods Validation

The improved reverse phase, high performance liquid chromatography concept's linearity, accuracy, precision, detection limit, and quantification limit were tested in order to validate it. To make sure the technique was appropriate for the quantitative measurement of benzoic acid in the food goods being studied, these factors were assessed (Huy & Hanh, 2026).

Linearity

Eleven calibration levels (1.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0, and 50.0 µg.mL^{-1}) were utilized to assess the linearity of the suggested approach over the concentration range of 1.0–50.0 µg/mL. The calibration curve was created using the mean peaks areas after each concentration was injected five times. The equation y = 19638.73x + 984.65, with R^2 = 0.9997, was obtained by linear regression analysis is showed in Figure 5 and Figure 6. The regression line's intercept was 984.65, and its slope was 19638.73. The discrepancies between the observed and anticipated peak regions were used to compute the residuals. The residual values, together with the corresponding mean peak areas, standard deviations, and relative standard deviations, are showed in the Table 6, The residual plot is showed in Figure 7. Both positive and negative residuals were observed around the zero line; however, some sequential patterns and deviations were evident, indicating that the suitability of the linear regression model should be considered in addition to the coefficients of determination.

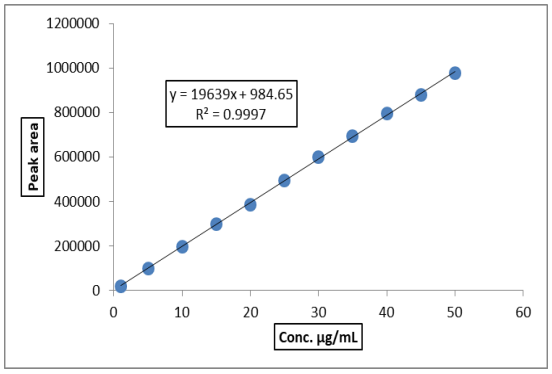


Figure 5: Calibration Curve for Benzoic acid under chromatographic conditions

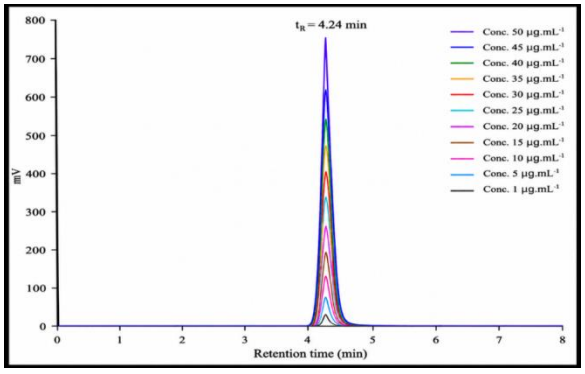


Figure 6: Peaks for different concentrations of Benzoic acid under chromatographic conditions

Table 6: The Residual Values, Together with the Corresponding Mean Peak Areas, SD, and RSD

Conc. (µg/mL)	Peaks areas means	S.D.	R.S.D.	Residual
1	18,751	2.55	0.01360	−1,872.38
5	97,766	2.55	0.00261	−1,412.31
10	196,641	2.24	0.00114	−730.96
15	298,330	1.87	0.00063	+2,764.38
20	385,155	1.73	0.00045	−5,504.28
25	493,891	2.35	0.00047	+1,938.07
30	598,783	2.92	0.00049	+5,436.41
35	693,460	2.35	0.00034	+5,119.75
40	794,301	2.35	0.00030	+4,637.10
45	878,781	1.73	0.00020	−5,946.56
50	975,262	1.58	0.00016	−4,519.21

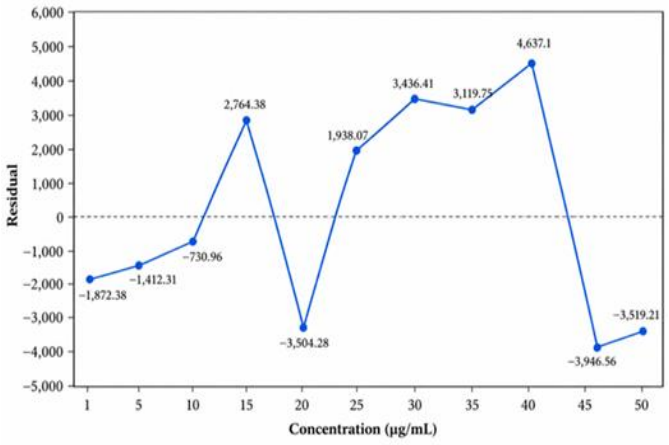


Figure 7: Residual plot for Calibration Curve

Accuracy and Recovery

Using samples of carbonated soft drinks and canned fruit juice, recovery experiments were conducted at three concentration levels to assess the accuracy of the suggested procedure. The native concentrations in each unspoiled samples were tested and deducted from total concentrations measures in matching spiked sample because benzoic acid was initially present in both food matrices. The obtained data show the recovered concentrations of the added benzoic acid. After dividing the recovered concentration by the corresponding added concentration, the result was multiplied by 100. The recovery values ranged from 98.88% to 101.55%, with RSD values ranging from 0.58% to 1.25%, as Table 7 demonstrates. These findings show that the recommended method has useful accuracy and precision in the food matrices under investigation Variations in matrix composition, including sugars, organic acids, and other food components, may affect chromatographic reaction and sample preparation, which could account for the small variations in recovery percentages.

Table 7: The Suggested RP-HPLC Method's Accuracy and Precision for Measuring Benzoic Acid in Contaminated Food Samples

Sample	Native conc. (µg/mL)	Added conc. (µg/mL)	Rec. conc. (µg/mL)	Rec. (%)	RSD (%)
Canned fruit juice	2.0	10	9.912 ± 0.081	99.12	0.82
Canned fruit juice	2.0	20	20.090 ± 0.225	100.45	1.12
Canned fruit juice	2.0	40	40.408 ± 0.271	101.02	0.67
Carbonated soft drink	2.5	10	9.888 ± 0.124	98.88	1.25
Carbonated soft drink	2.5	20	19.952 ± 0.188	99.76	0.94
Carbonated soft drink	2.5	40	40.620 ± 0.236	101.55	0.58
Tomato paste	3	35	.3545 ± 0.244	101.29	0.69

Precision and Accuracy

Replicate measurements were conducted on the same day under identical experimental settings to measure the precision of the suggested method in terms of repeatability. The relative standard deviation (RSD%) was used to express precision. RSD values below 1.25% in the data demonstrated the method's strong reproducibility. The recovery studies with samples of carbonated soft drinks showed the highest RSD value of 1.25%. These results show that repeated measurements under given experimental condition are consistent. These study did not assess intermediate precision.

Detection Limit and Quantification Limit

The residual standards deviations of the calibration curve and its slope were used to detects the limits of detection (LOD) and quantification (LOQ). The following equations were used to conduct the calculations:

(L.O.D) = 3.3 σ/s

(L.O.Q) = 10 σ/s.

where S is calibration curve's slope and σ is regression's residual standard deviation. The slope was 19638.73 peak-area units per µg/mL, and the residual standard deviation was 6205.90 peak-area units based on the calibration data. As a result, the LOD and LOQ were determined to be 1.04 and 3.16 µg/mL, respectively.

Determination of Benzoic Acid in Commercial Food Samples

After making adjustments and verifying the results, RP-HPLC technique invented was tested on selected commercial food samples purchased from the community marketplace. Samples consisted of carbonated drinks, fruit juice drinks and processed tomato paste, as shown in Table 8.

Table 8: The Measured Concentrations of Samples

Food Sample	Benzoic Acid Concentration
Orange juice	120.5 ± 1.4 mg/L
Cola drink	145.0 ± 1.1 mg/L
Tomato paste	45.2 ± 0.9 mg/kg
Apple nectar	ND*

ND: Not detected under the analytical conditions of the method.*

Note: Regulatory limits were obtained from the Codex General Standard for Food Additives (CXS 192-1995) and the Codex Standard for Processed Tomato Concentrates (CXS 57-1981).

The results of the analysis of selected food samples from the products revealed some variation in the findings; This can be explained by variations in the makeup of each product, the quantity and type of preservatives added during the manufacturing process, processing conditions and acidity. Nevertheless, the findings indicated that the estimated concentrations for benzoic acid were close to the correct limits, which vary due to the type of food to which it is added and differ for each individual product; there is no single limit that applies to all products.

Conclusion

For the purpose of determining the quantities of benzoic acid in particular commercial food products, Reversed phase HPLC-UV method was created and assessed. A C18 column, methanol: water mobile phase (40:60, v/v) adjusted to pH 3.5, a flow rate of 1.0 mL per min, and UV detector at 228 nm were the chosen chromatographic conditions. Benzoic acid showed a retention duration of roughly 4.24 minutes under these circumstances. With a coefficient of determination of 0.9997, the technique demonstrated a linear curve response between 1.0–50.0 µg/mL. The recovery values for the studied beverage matrices varied from 98.88% to 101.55%, with stated detection and quantification limits of 1.04 and 3.16 µg/mL, respectively. Benzoic acid concentrations varied noticeably amongst the products under investigation when applied to commercial samples. These results imply that the technique may be useful for analyzing benzoic acid in specific dietary matrices. However, before the method can be suggested for thorough routine food-safety monitoring, more clarity on extraction procedures, chromatographic selectivity, matrix-specific validation, and acceptable and regulatory standards is required.

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