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Determination of Benzoic acid Residue from Fruit Juice by Gas chromatography with Mass spectrometry Detection Technique

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ABSTRACT

A simple and reliable Gas chromatography-Mass spectrometer method was developed for the determination of Benzoic acid in fruit juice. Chloroform was used for extraction solvent and co-extractive was removed by ether and 0.5M NaOH. The method was validated by determining parameters such as, specificity, linearity, limit of detection, limit of quantitation, precision, recovery and robustness. The method was found to be specific against fruit matrix interferences. Linearity was evaluated over the concentration ranges of 0.1 µg/ml to 10 µg/ml and correlation coefficient was more than 0.999. Both the inter day and intra day precision of the system and method were determined. Recovery data obtained by fortifying three matrices at 1.0 µg/g and 2.0 µg/g with ranged between 98 to 105 % and the relative standard deviation (RSD) was obtained below 5%. Limit of detection and quantitation of benzoic acid were 0.05 and 0.1 µg/g.

Keywords: Benzoic acid, Preservative, Fruit juice, Gas chromatography-Mass spectrometer.

INTRODUCTION

Benzoic acid (CAS No. 65-85-0); molecular formula $C_7H_6O_2$; C_6H_5COOH, IUPAC name: Benzene carboxylic acid or Phenyl carboxylic acid; molecular weight 122.13, is a white solid that starts to sublime at 100°C, with melting point of 122°C and boiling point of 249°C. Benzoic acid is used as food preservatives and is most suitable for foods, fruit juices and soft drinks that are naturally in an acidic pH range. Benzoic acid also used as preservative in food, beverages, tooth pastes, mouth washes, dentifrices, cosmetics and pharmaceuticals is regulated. The estimated global production capacity for benzoic acid is about 6, 00,000 tones per year. Benzoic acid occurs naturally in many plants and animals. It is therefore natural constituent of many foods, including milk products. Anthropogenic releases of benzoic acid into the environment are primarily emissions into water and soil from their uses as preservatives. Concentration of

naturally occurring benzoic acid in several foods did not exceed average values of 40mg/kg of food. Analytical methods for determination of benzoic acid include spectrophotometric methods, which need extensive extraction procedures and are not very specific, gas chromatographic (GC) and high performance liquid chromatographic (HPLC) methods, which has high sensitivity and specificity [1-10]. Halvorson et.al (1984) reported a direct determination of benzoic acid in air by flash desorption at 240°C with helium into capillary-GC gave a detection limit of 0.1 ppm (0.5mg/m^3) in a 20 liter sample (10µg benzoic acid). This method has been developed and used for monitoring occupational exposure [11]. A method (Larsson, 1983) for the determination of benzoic acid in solid food at 0.5-2 g/kg levels involves extraction with ether into aqueous sodium hydroxide and Methylene chloride, conversion to trimethylsilyl esters and detection by GC and flame ionization [12]. Determination of benzoic acid in margarine was extracted with ammonium acetate/acitic acid/methanol by using HPLC and Ultra violet detection, has been described by Arens & Gretz 1990 [13].

In this paper we validate and described the extraction procedure carried out with chloroform. For determination of Benzoic acid in fruit juice samples, GC/MS with Electron ionization mode was used to simultaneously identify and quantify benzoic acid in food matrix.

MATERIALS AND METHODS

Reagents and chemicals

Chloroform, Ether (HPLC grade) was purchased from SD fine chemicals, Mumbai, India. Reagent grade anhydrous sodium sulfate, sodium chloride, sodium hydroxide were obtained from SD fine chemicals, Mumbai, India. HCl and H_2SO_4 with AR grade were purchased from SD fine chemicals, Mumbai. Benzoic acid with purity >99% were obtained from Fluka chemicals, USA. Fruit juices were obtained from local market of Delhi (India).

GC/MS analysis

Gas chromatography analysis was carried out using Agilent Technologies 6890 N network GC system, equipped with a Agilent Technologies 7683 series auto sampler, Mass selective detector Model 5973 network, and a glass capillary DB-5, ID: 0.32 mm, length: 30 m, film thickness: 0.25 µm; packed with non-polar polymer [(5%-phenyl)-methylpolysiloxane] (J & W Scientific 122-5532, USA).; a helium carrier gas flow. 1.0 ml/min; injection temperature 280°C; transfer line temperature 300°C, ion source temperature 230°C, MS Quard temperature 150°C; ion mode electron ionization mode (scan mode/SIM mode); oven temperature program 50°C for 2min, @ 10°C/min to 180°C, post run: 280°C held for 5 min; splitless injection at a volume of 1 µl by auto sampler.

Mettler weighing balances with a least count of 0.0001g and 0.001mg for weighing of samples and standards respectively. Calibrated ‘A’ grade glassware of Borosil procured from local market.

Preparation of 0.5M NaOH (Sodium hydroxide).

Approximately 2 g of sodium hydroxide was accurately weighed and transferred into 100 ml volumetric flask dissolved and diluted to volume using HPLC grade water.

Preparation of Calibration standard solutions:
Stock solutions of Benzoic acid at 100µg/ml was prepared by dissolving 2.5 mg benzoic acid standard in chloroform and made up to 25 ml and storing the solution cool and dark place.

Extraction:
Approximately 5±0.1 g of homogenized test sample (fruit juice) was weighed accurately and transferred into the 50 ml centrifuged tube with Teflon-lined screw cap. To this 1.5 ml H_2SO_4 (1:5), 5 g sand and 15 ml ether were added and screw cap was on tightly to avoid leakage. Mechanically shaked for 5 min and centrifuged at 1500 × g for 10 min. Transferred ether layer by disposable pipette into 250 ml separatory funnel. Extraction was repeated twice with 15 ml ether each time. Extracted combined ether phase twice with 15 ml 0.5 M NaOH and 10 ml saturated NaCl solution each time. Collected the aqueous layer into the 250 ml separatory funnel, added 2 ml of methyl orange and maintained pH 1 by acidified with HCl (1:1). Extracted the aqueous layer thrice with 75 ml of $CHCl_3$ each time and passed through filter contained 15 g anhydrous Na_2SO_4 into 250 ml round-bottom flask and evaporated $CHCl_3$ solution in rotary evaporator at 40°C, made the dilution to 5 ml.

Method validation:
The method was validated for the determination of Benzoic acid for the following parameters: specificity, linearity, precision, accuracy, limit of detection, limit of quantitation, robustness and ruggedness as per the ICH (International Conference on Harmonization) guidelines.

Specificity: The specificity of the method was determined by analyzing the benzoic acid standard solution with the sample solution fortified with standard stock solution and blank juice sample. For this purpose 1µl of spiked sample solutions and standard solution were injected into the gas chromatograph with mass spectrometer and the specificity of the method was measured in terms of the m/z value between the peaks arised from spiked sample solution and standard solution.

Linearity: Linearity of the method was determined by plotting a calibration curve for benzoic acid for concentration Vs detector response (area counts in mV). For this purpose, Calibration standard solutions were prepared by diluting the stock solution (100 µg/ml) at five levels in the ranged 0.1, 0.5, 1.0, 5.0 and 10.0µg/ml and for matrix match calibration fortifying the sample by spiking known concentration of working standard solution. From each of these calibration standards 1µl was injected into the GC-MS. The calibration curve obtained was subjected to regression analysis by the least square method to calculate the calibration equation and the correlation coefficient (r).

Limit of Detection and Limit of Quantitation: For the measurement of limit of detection, standard deviation of the background was determined. Thereafter, standard solutions of very low concentration levels i.e. 0.05µg/ml, 0.1µg/ml and 0.2 µg/ml were injected till the signal obtained was thrice the standard deviation of the background.

For the measurement of limit of quantitation again, known concentration of standard benzoic acid solutions were injected till the signal obtained was reproducible for five replicate injections.

Precision: The precision of the method was determined in terms of repeatability or reproducibility and intermediate precision studies. Repeatability was determined by evaluating five replicates of the three different concentrations i.e. 0.1 µg/ml, 0.5 µg/ml and 5.0 µg/ml of the calibration standard solution of benzoic acid on the same day (intra-day) under the mentioned chromatographic conditions. The intermediate precision of the method was assured by performing the analysis on three different days (inter-day) and also by different analysts in the same laboratory (between analysts). Co-efficient of variation or the percent RSD was calculated in each case.

Recovery study: The accuracy and recovery study of the method was evaluated by spiking different known concentrations of benzoic acid into the pre-analyzed juice samples which was carried out by adding 0.05 ml and 0.10 ml of 100 µl/ml stock solution into the 5g of each sample, given concentration 1µg/g (1-ppm) and 2 µg/g (2-ppm). 1µl of each of these solutions of five replicate was injected onto GC-MS and the closeness of the results to the true value was determined.

Robustness: Robustness of the method was determined by analyzing the same sample under different conditions of method parameters such as two different makes of gas chromatograph, same column from two different manufacturers, different analysts, and varying injection volume.

RESULT AND DISCUSSION

Selectivity/specificity: The selectivity or specificity of analytical method for benzoic acid in fruit juice is represented in Figure.1. The chromatogram indicates that the developed method was successful in separation of benzoic acid in complex food matrix. The peak of the chromatograms is also confirmed by the m/z value of standard benzoic acid with NIST library of GC-MS (Figure 2). The peak area of benzoic acid obtained the RSD value was within 5% at ppb level. The retention time had good reproducibility, within RSD 0.05%. Table 1 shows the results for selectivity and specificity of benzoic acid in fruit juice.

Linearity and Range: The calibration curve for benzoic acid was prepared by plotting peak area against the concentration of benzoic acid in fruit juice matrix and in blank solvent. It was linear in the range of 0.1µg/ml, 0.5 µg/ml, 1.0 µg/ml, 5.0µg/ml and 10.0 µg/ml gave linear response over the studied range of concentration, and the least squares linear regression analysis of the data provided excellent correlation coefficient (r) of more than 0.999 for both calibration standard in juice matrix and blank solvent. The linear equations, correlation co-efficient and RSD values are presented in Table 2.

Limit of detection (LOD) and limit of quantitation (LOQ): LOD was determined by considering signal to noise (S/N) ratio of 3:1 for the strongest mass transition with respect to the background noise obtained from the blank sample whereas LOQ was determined similarly by considering signal to noise ratio (S/N) ratio of 6:1. Based upon the mean noise level for the ten injections of the matrix blank of fruit juice sample, lowest detection limit of the instrument was calculated as 0.05µg/g and confirmed using standard solutions with concentration of 0.05µg/g and the lowest concentration levels that could be quantified with reproducible values obtained on injecting 6

replicates of the same concentration as 0.1µg/g and further confirmed by injecting 6 replicates of matrix matched standard solution of benzoic acid having concentration of 0.1µg/g.

Precision: Precision studies were carried out for both intra-day and inter-day repeatability and reproducibility (Table 3). Spiked sample of fruit juice at different concentration levels i.e. 0.1 µg/g, 0.5 µg/g and 5.0 µg/g respectively were injected five replicates on the same day and the same number of times on three subsequent days by three different analysts. The low %RSD value obtained for intra-day and inter-day variation within the acceptable norms showed that the proposed method is precise and can be adopted for analysis.

Table 1: Selectivity, specificity and LOD & LOQ of Benzoic acids and general GC/MS information

Compounds	Category	R.T (min)	Parent ion, m/z	Product ions, m/z	LOD (µg/g)	LOQ (µg/g)
Benzoic acid	Preservative	10.1	105	122, 77	0.05	0.1

Table 2: Comparison of matrix matched calibration with standard calibration, and repeatability data for the Benzoic acid.

Standard	Matrix calibration			Solvent calibration			Slope standard/ slope matrix	Repeatability (% RSD)
	Slope	y-intercept	R^2	Slope	y-intercept	R^2		
Benzoic acid	14016	-6416	0.999	14446	-9504	0.999	1.030679	3.57

Table 3: Intra-day and Inter-day precision data for the proposed method for Benzoic acid residues in three samples of fruit juices

Concentration of Benzoic acid (µg/g)	Sample No	Day 1		Day 2		Day 3		Intra-assay	
		Benzoic acid conc. obtained (µg/g) n=5	% RSD	Benzoic acid conc. obtained (µg/g) n=5	% RSD	Benzoic acid conc. obtained (µg/g) n=5	% RSD	Benzoic acid conc. obtained (µg/g) n=5	% RSD
0.1	1	0.100	3.72	0.104	3.55	0.103	3.65	0.102	3.62
	2	0.101	3.56	0.102	3.48	0.099	3.38	0.101	3.55
	3	0.099	3.82	0.100	3.21	0.101	3.46	0.099	3.28
0.5	1	0.503	2.82	0.501	2.76	0.504	2.92	0.501	2.71
	2	0.500	2.75	0.502	2.86	0.502	2.85	0.500	2.82
	3	0.499	2.12	0.501	2.81	0.500	2.69	0.499	2.52
5.0	1	5.06	1.25	5.05	1.42	5.09	1.21	5.05	1.35
	2	5.08	1.31	5.04	1.34	5.05	1.60	5.07	1.27
	3	5.00	1.02	4.99	1.38	5.01	1.52	5.00	1.20

Recovery study: The recovery of benzoic acid in spiked samples was calculated to study the effect of matrix on the determination of benzoic acid. The recovery studies were carried out at two different concentrations. For this two different portions of pre-analyzed three different fruit juice samples were spiked with 1.0 µg/g and 2.0 µg/g respectively in six replicates on three different days and then extracted and determined by the same method as mentioned earlier. The recoveries of benzoic acid from the fruit juice samples were evaluated on the basis of the comparison of the theoretical concentration level of the spiked solutions with the observed

concentration gave acceptable and good percent recoveries found in the range of 98% to 105% are shows in Table 4.

Table 4: Percent recovery of Benzoic acid from three different fruit juice samples analyzed on different days (n=6)

Spiking level µg/g	Sample No 1	Day1		Day 2		Day 3	
		Amount calculated µg/g	% Recovery	Amount calculated µg/g	% Recovery	Amount calculated µg/g	% Recovery
1.0	1	0.971	97.1	1.031	103.1	0.999	99.9
	2	0.982	98.2	1.024	102.4	1.012	101.2
	3	0.991	99.1	0.998	99.8	1.041	104.1
2.0	1	2.06	103.0	1.98	99.0	2.02	101.0
	2	2.04	102.0	1.99	99.5	2.01	100.5
	3	2.01	100.5	2.00	100.0	2.02	101.0

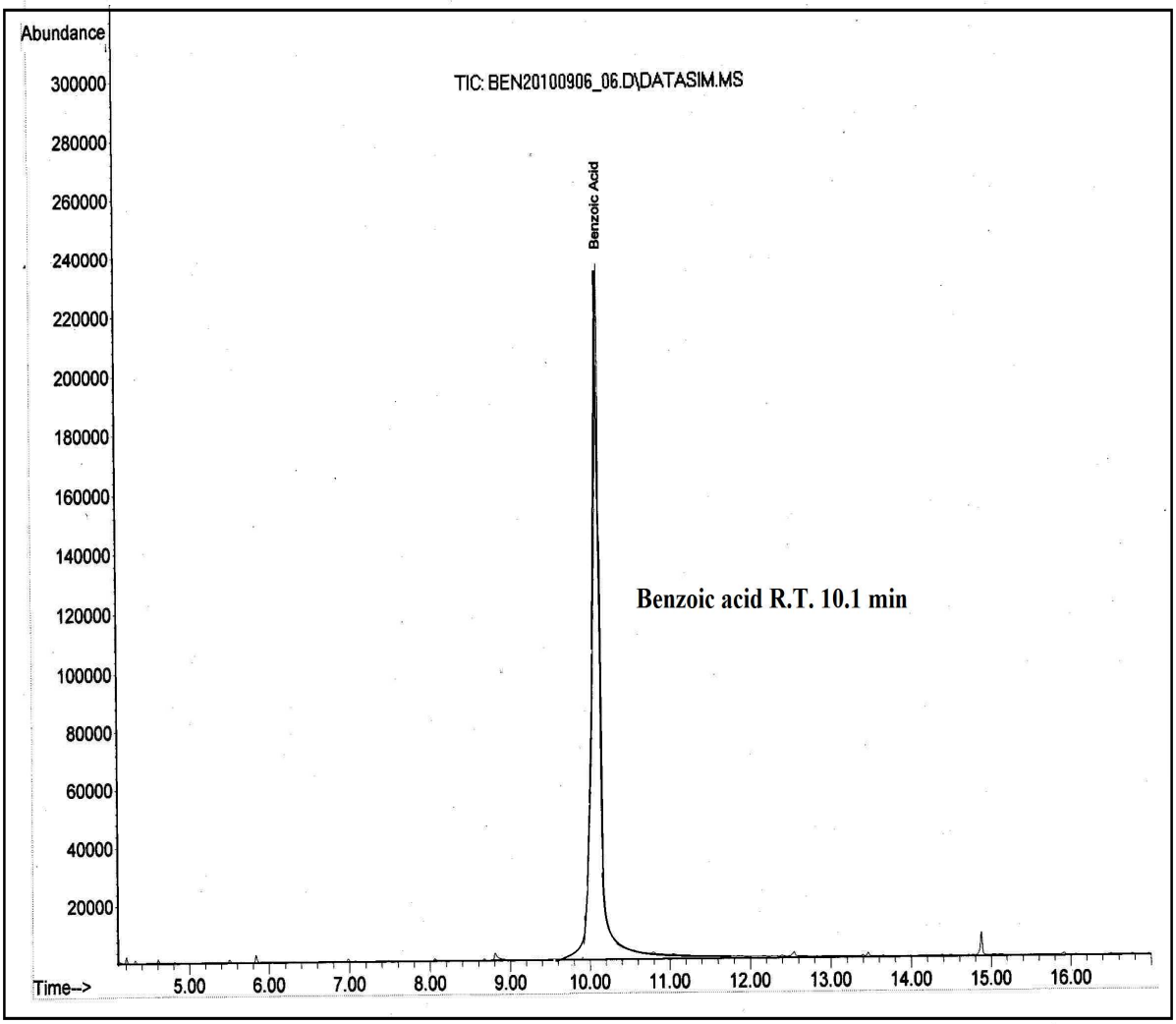
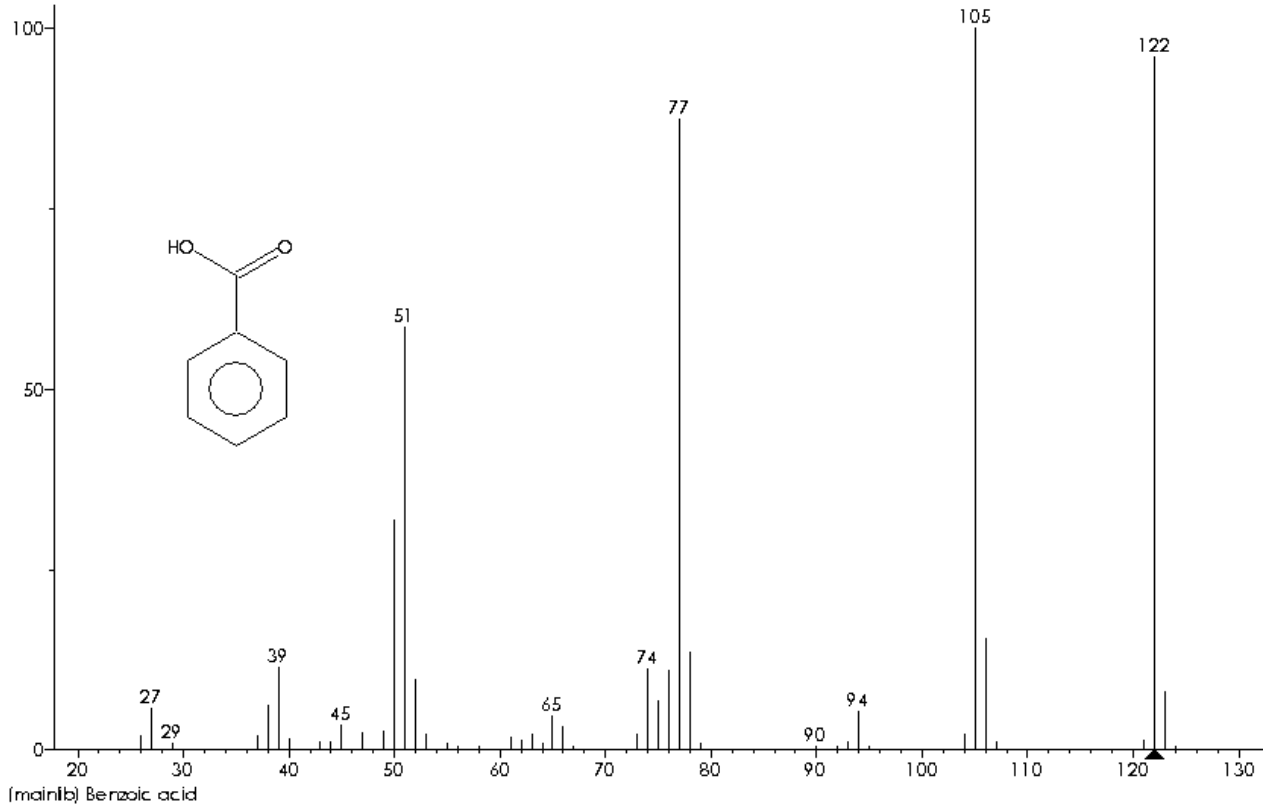


Figure 1: Total Ion Chromatogram of Benzoic acid in Fruit juice sample



Benzoic acid

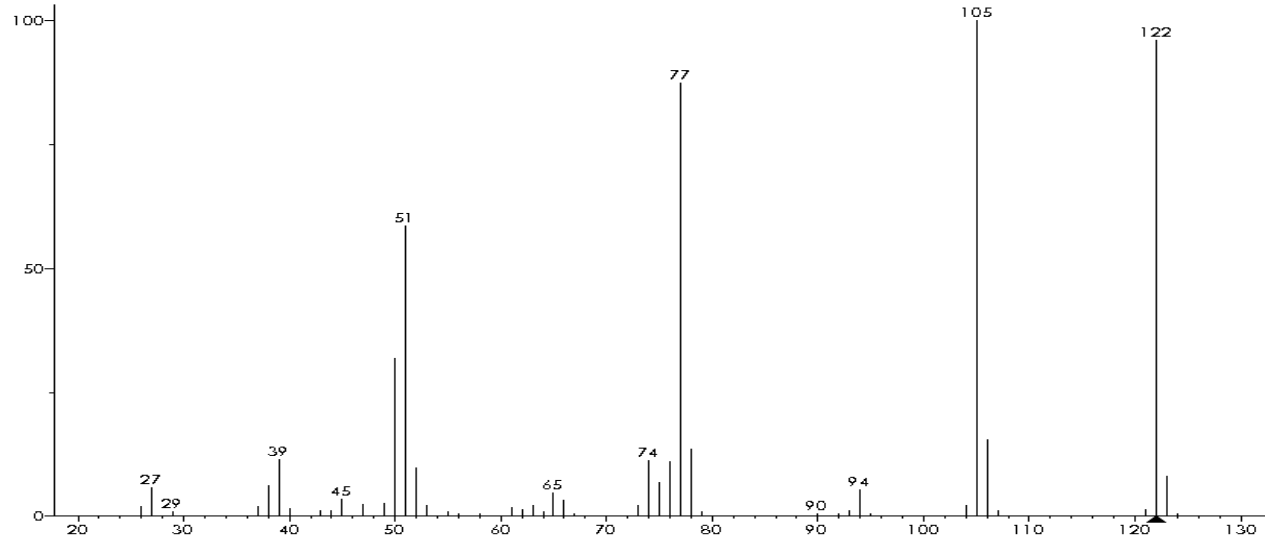


Figure 2: Mass Spectra of Benzoic acid in the Fruit juice sample and matching form NIST Library.

R*obustness:* Robustness of the method was determined by analyzing the same set of spiked samples (i.e. samples spiked at concentration levels of 0.1 µg/g, 0.2 µg/g and, 0.5 µg/g) under different parameters; such as same column chemistry from different manufacturers, different analysts, and different injection volumes. The method was found to be robust even with small changes in analytical conditions: change in flow rate (± 0.1 ml/min), a change in injector temperature (± 2°C), use of same column from different manufacturers (HP5, DB5, CP-sil 8 CB). Under all of these conditions, the analytical values of the spiked samples were not affected and it was in accordance with the actual values.

CONCLUSION

Benzoic acid is used as a preservative in fruit juice but it should be present at ppm level because at high concentration its affects to the human health. A validated gas chromatography-Mass spectrometer method has been developed for the determination of benzoic acid in fruit juice at ppm level. The work described in this paper has shown that the analytical method developed is precise, accurate, sensitive and robust for the determination of benzoic acid. The method is specific to the analysis of benzoic acid in fruit juice without any matrix interference.

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