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DEVELOPMENT AND VALIDATION OF A HPLC METHOD FOR THE QUANTIFICATION OF BENZOYL PEROXIDE IN FLOUR

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ABSTRACT

An isocratic high performance liquid chromatographic method has been developed and validated for the estimation of benzoyl peroxide in flours.The benzoyl peroxide was extracted with acetonitrile and analysed by Waters HPLC instrument on a C18 column using water-acetonitrile mobile phase with a flow rate of 1.5 ml/min and UV detection at 235 nm.The linearity was found in the range of 5-50 mg/l.The method is simple,reliable, specific,accurate and precise.

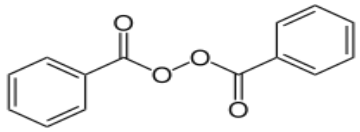
KEY WORDS: ***Benzoyl peroxide, Acetonitrile, HPLC, Method Development,Method Validation,.***

ABBREVIATIONS

BP-Benzoyl Peroxide,HPLC-High performance Liquid Chromatography,PDA-Photo Diode Array,IARC-International Agency for Research on Cancer,WHO-World Health Organization,FAO-Food and Agriculture Organization,FSSAI-Food Safety and Standards Authority of India,QC-Quality Control,LoD-Limit of Detection,LoQ-Limit of Quantification,RT-Retention Time.

1. INTRODUCTION

Benzoyl peroxide,otherwise called benzoyl superoxide,is an organic compound with chemical formula $C_{14}H_{10}O_4$.It's a colourless crystalline powder insoluble in water.



It's a food additive(INS 928) that can be used as a flour treatment agent.

Freshly milled flours have an yellowish appearance due to the presence of carotenoids in them.Benzoyl peroxide is a bleaching agent and promotes the oxidation of carotenoids and whitens the flour as a result.It is often used as some customers prefer white flour to yellowish ones.

The Joint WHO/FAO Expert Committee on Food Additives has noted that when benzoyl peroxide is used as a bleaching agent in flour,it reacts with the oxidizable substances that are present and is converted into benzoic acid,a commonly used preservative.The committee concluded that the intake of benzoic acid from food stuffs treated with benzoyl peroxide should be considered together with other dietary sources of benzoates in the group ADI of 0-5 mg/kg of body weight.

An excessive amount of benzoyl peroxide not only annihilate the nutrients in flour but also affects human health.However,IARC not classified benzoyl peroxide as a carcinogen.

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2. REGULATIONS FOR BENZOYL PEROXIDE

Table-1 : Limit for BP under FSSAI regulation

Sl No	Type	Maximum permissible limit (mg/kg)
1	Maida	40
2	Bread and ordinary Bakerywares and mixes	80
3	Cakes,cookies,biscuits,crackers and pies	40
4	Atta	Not permitted
5	Other flour and starch	75

The Codex Alimentarius Commission(2021) has determined that the amount of benzoyl peroxide allowed in flour cannot be greater than 75 mg/kg.In India,the regulator FSSAI notified the Food Safety and Standards(Food Products and Food Additives) Regulation,2011 which stipulates limits for different products(Table-1).

3. MATERIALS AND METHOD

3.1. Chemicals

Benzoyl peroxide (97% purity) was obtained from A2S.Milli-Q Water and LC-MS grade Acetonitrile were used.

3.2. Instrumentation

The detection wavelength was determined by using Shimadzu 2600 UV-Visible spectrophotometer.The LC system used for method development and validation consists of a Waters 2695 series HPLC system equipped with PDA detector (2996).Data acquisition,analysis and reporting were performed by Empower2 software. Electronic balance(ACZET) of resolution 0.00001 g,Sonicator, Centrifuge(REMI) and Vortex(SPINIX) were also used in this study,performed at Regional Analytical Laboratory , Calicut , Kerala during August 2023.

4. EXPERIMENTAL

4.1.Method Development

4.1.1 Preparation of Stock and Working Standard Solutions

The stock standard solution of BP was prepared in acetonitrile at 200 mg/l concentration.The working standard solutions at six different levels (5,10,15,20,25,50 mg/l) were prepared by serial dilution of the stock solution in acetonitrile.

4.1.2 Preparation of Quality Control(QC) solutions

The QC stock solutions of BP independent of the standard stock solution was prepared in acetonitrile.Then QC sample solutions (15 and 20 mg/l) were prepared by serial dilutions of QC stock standard solution in the same diluent.

4.1.3 Selection of wavelength

To determine the wavelength for measurement,BP working standard solution (25 mg/L) was scanned in UV-VIS spectrophotometer in the range of 200-400 nm against acetonitrile as blank.The maximum absorbance was observed at 235 nm.

4.1.4 Preparation of Mobile Phase

Mobile phase was prepared by mixing 55 volumes of acetonitrile and 45 volumes of water.The prepared solution was sonicated and filtered through 0.45 µM membrane filter.

Table-2: Optimized parameters

Parameter	Condition
Column	Waters® C18(250mm x 4.60 mm ID , 5 µM particle size)
Mobile phase	45:55 (v/v) Water-Acetonitrile
Flow Rate	1.5 ml/min
Wavelength	235 nm
Injection Volume	20 µL
Run time	20 min
Retention Time	Approx. 13.8 min

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4.1.5 Chromatographic condition

The chromatographic separation was performed using isocratic elution at 40^{O}C on Waters® C18 column (250mm x 4.60 mm ID , 5 µM particle size) and detection wavelength of 235 nm.The elution was monitored by injecting the 20 µL and the flow rate was adjusted to 1.5 ml/min.

4.1.6 Sample Extraction Procedure

Add 25 ml acetonitrile to 5 grams homogenized sample in an extraction tube.The mixture was,then, vortexed 5 minutes and centrifuged at 4500 rpm for 10 minutes at 25^{O}C.The supernatant was collected,filtered through 0.45 µm syringe filter and an aliquot of 20 µL was used for injection.

4.1.7 Robustness

Robustness of an analytical procedure/method is a measure of its capacity to remain unaffected by small but deliberate variations in the procedural parameters listed in the documentation, indicating the suitability or reliability of the method during normal use.This can be done by small deliberate change in the internal factors of the method related to sample preparation, mobile phase composition,mobile phase flow rate, injection volume,column temperature etc.

No deliberate change in the chromatogram was observed when the flow rate (± 0.1 ml/min) and column temperature (±2 degrees) are changed.

4.2 Analytical Method Validation

The developed method was validated by single laboratory approach.The following method performance characteristics were evaluated.

4.2.1 Specificity/Selectivity

Analytical selectivity relates to 'the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behaviour'.It is investigated by comparing the chromatogram of benzoyl peroxide standard solution (25 mg/l) with that of blank sample(acetonitrile).The blank has no interfering peak at the retention time of benzoyl peroxide.

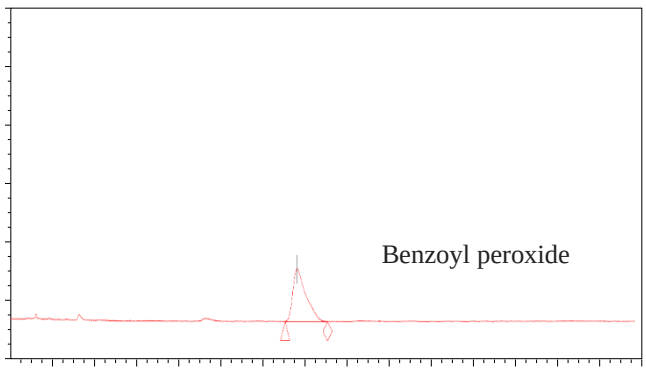

Fig-1 : Chromatogram of BP (25 mg/l)

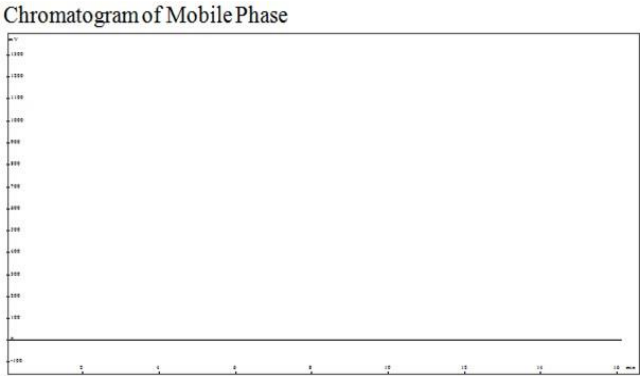

Fig-2 : Chromatogram of mobile phase

4.2.2 Linearity

The linearity is the ability of the method to obtain test results that are directly proportional to the analyte concentration,within a specific range.

The linearity was established by using a series of standard solutions of benzoyl peroxide,studies repeated in three replicates.The calibration curve ,obtained by concentration on X-axis against peak mean area on Y-axis, showed linearity in the concentration range of 5 to 50 mg/l.

Regression equation was**: Y = 96497x – 23554**. Co-relation coefficient was: **R^2 = 0.9999**,

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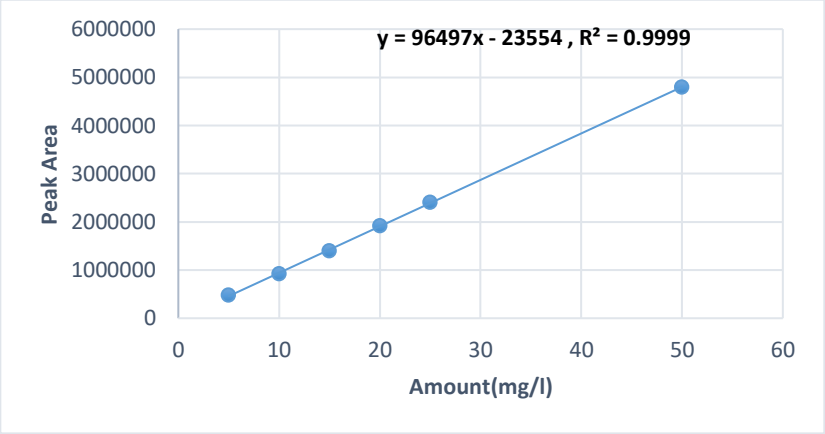

Fig-2: Linearity curve

Sl No	Concentration (mg/L)	Peak Area
1	5	479590.344191
2	10	927653.007965
3	15	1397258.94043
4	20	1915256.009147
5	25	2400337.670830
6	50	4800675.332678
	Correlation coefficient (R2)	0.9999
	STEYX	19599.58
	Slope	96497

Table-3: Linearity & Statistical analysis

STEYX is a function used to calculate standard error.

4.2.3 LoD and LoQ

The LoD and LoQ were determined from the linearity curve.

$$\textbf{LoD} = \frac{\textbf{STEYX} * 3.3}{\textbf{SLOPE}} \qquad \text{LoD} = 0.670266$$

$$\textbf{LoQ} = \frac{\textbf{STEYX} * 10}{\textbf{SLOPE}} \qquad \text{LoQ} = 2.031108$$

Here,the lowest calibration point is considered as LoQ. Thus **LoQ = 5.0 mg/l**

4.2.4 Recovery Studies

The accuracy of the method determines the closeness of results obtained by that method to the true value.

The recovery experiments were carried out to study the accuracy and reproducibility of the proposed method.A fixed amount of pre-analyzed rice powder samples were taken and the standard benzoyl peroxide was added at LoQ,2LoQ and 5LoQ levels.Each level was repeated in five times.The results are shown in Table-4.

Table-4 : Recovery

Level	Sl No	Amount added	Amount Recovery	% Recovery	Mean Recovery	Recovery Std Deviation	% RSD
LoQ	1	5.0	5.891	117.82	116.496	2.7008	2.32
	2	5.0	5.903	118.06			
	3	5.0	5.613	112.26			
	4	5.0	5.946	118.92			
	5	5.0	5.771	115.42			
2LoQ	1	10.0	11.096	110.96	108.37	2.0575	1.9
	2	10.0	10.557	105.57			
	3	10.0	10.76	107.6			
	4	10.0	10.967	109.67			
	5	10.0	10.805	108.05			
5LoQ	1	25.0	25.579	102.316	103.0112	0.6363	0.62
	2	25.0	25.818	103.272			
	3	25.0	25.603	102.412			
	4	25.0	25.808	103.232			
	5	25.0	25.956	103.824			

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4.2.5 Precision

The precision of analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.The precision may be considered at three levels;repeatability(Table-5),reproducibility(Table-6) and RT repeatability(Table-7).

4.2.6 Ruggedness

Ruggedness is "the degree of reproducibility" of the test result when external factors such as analyst, laboratory, instrument, reagents and days are varied.In this single laboratory validation,ruggedness was done by changing the analyst. The results indicate that the selected factors are remained unaffected by small variations of this parameter.(Table-8)

Sl No	Concentration (mg/l)	Peak Area
1	15.0	1405004.01
2		1421587.25
3		1393404.01
4		1422663.85
5		1422591.99
Average		1413050.22
Standard Deviation		13295.33
% RSD		0.9409

Table-5 : Repeatability(Intra Day Precision)

Sl No	Concentration (mg/l)	Peak Area
1	20.0	1912278.50
2		1892434.01
3		1893820.99
4		1907294.00
5		1899267.00
Average		1901019
Standard Deviation		8583.922
% RSD		0.3545

Table-6 : Reproducibility(Inter Day Precision)

Sl No	Concentration (mg/l)	Retention Time (min.)
1	5.0 mg/l	13.876
2		13.821
3		13.834
4		13.839
5		13.855
Average		13.845
Standard Deviation		0.021177819
% RSD		0.15

Table-7 : Retention Time Repeatability(Precision)

Concentration(mg/l)	Analyst-1	Analyst-2
25.0	2402056.00	2377152.52
	2409834.00	2378389.01
	2630164.50	2400337.67

Table-8 : Ruggedness by different analyst

5. STABILITY OF ANALYTICAL SOLUTIONS

To evaluate stability of samples during the analysis, stability of 2 levels (15 mg/l and 20 mg/l) QC samples under 3 different conditions were checked by replicated analysis (N = 3). After short-term storage (at 25 °C for 24 h), after long-term storage (at 2-8 °C for 2 weeks) and after going through three freeze-and-thaw cycles (from -20 to room temperature for every 24 h), aged QC samples reanalyzed versus freshly prepared standard solution. Based on these results, QC samples are considered stable for 24 h at room temperature, up to 14 days when stored under refrigeration and after three freeze-and-thaw cycles.

Storage Condition	Theoretical concentration(mg/l)	Calculated Concentration(mg/l)	Precision (% RSD)	Accuracy (% Recovery)
Short term	15	15.0959 ± 0.001	0.0095	100.64
	20	19.9690 ± 0.001	0.0048	99.84
Long term	15	14.5871 + 0.002	0.0150	97.25
	20	19.0720 ± 0.003	0.0173	95.36
Freeze and thaw	15	14.7486 ± 0.002	0.0113	98.32
	20	19.8921 ± 0.002	0.0087	99.46

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6. CONCLUSION

Based on the result,it is concluded that isocratic RP-HPLC method was successfully developed for the estimation of benzoyl peroxide in flours.It is validated and give comparable results.The proposed method is suitable for routine analysis of benzoyl peroxide in flours.

7. CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

8. ACKNOWLEDGEMENT

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