DEVELOPMENT OF VALIDATED UV SPECTROSCOPIC AND CHROMATOGRAPHIC METHODS FOR THE ESTIMATION OF ISRADIPINE IN BULK DRUG AND BUTYLATED HYDROXYANISOLE IN SELECTED FOOD STUFFS

A Dissertation submitted to

THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY,

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In partial fulfilment of the requirements for the award of the Degree of

MASTER OF PHARMACY
IN
BRANCH–V- PHARMACEUTICAL ANALYSIS

Submitted by

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CERTIFICATE

This is to certify that the dissertation entitled **"Development of Validated UV Spectroscopic and Chromatographic Methods for the Estimation of Isradipine in Bulk Drug and Butylated Hydroxyanisole in Selected Food Stuffs"** being submitted to The Tamil Nadu Dr.M.G.R Medical University, Chennai was carried out by **Ms. S. SUMITHRA** in the **Department of Pharmaceutical Analysis,** College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, under the supervision and guidance of **Dr. A. Suganthi, M.Pharm., Ph.D.** Associate Professor, Department of Pharmaceutical Analysis, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore.

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Date:

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LIST OF ABBREVIATIONS

ACN	-	Acetonitrile
API	-	Active pharmaceutical ingredient
BHA	-	Butylated Hydroxyanisole
FDA	-	Food and Drug Administration
GRAS	-	Generally Recognized As Safe
gm	-	Gram
HPLC	-	High Performance Liquid Chromatography
HPTLC	-	High Performance Thin Layer Chromatography
ICH	-	International Conference on Harmonization
LOD	-	Limit of detection
LOQ	-	Limit of quantification
mg	-	Milligram
min	-	Minute
ml	-	Millilitre
μg	-	Microgram
μL	-	Microlitre
NFA	-	National food administration
R_f	-	Retardation factor
RP-HPLC	-	Reverse Phase High Performance Liquid Chromatography
RP-HPTLC	-	Reverse Phase High Performance Thin Layer Chromatography
RSD	-	Relative Standard Deviation
R_t	-	Retention time
SPAs	-	Synthetic phenolic antioxidants
T_f	-	Tailing factor
TOF- MS	-	Time of flight mass spectroscopy
λ_{max}	-	Wavelength of maximum absorbance

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INTRODUCTION [1-9]

Pharmaceutical analysis is a branch of chemistry, which involves the series of process for the identification, determination, quantitation and purification. This is mainly used for the separation of the components from the mixture and for the quantification of the compounds. [1]

The modern pharmaceutical analysis entails the following activities like,

- Analysis of drug substances in bulk drug
- Degradation and impurity analysis of drug substances
- Finger print analysis of harmful organic substances in food materials
- Food and cosmetic analysis
- Preformulation analysis
- Analysis of solid dosage forms
- Analysis of injectable dosage forms
- Development of new dosage forms
- Method development
- Method validation
- Biological analysis
- Kinetic studies
- Food - food interaction and food - drug interaction studies etc.

Analytical Techniques [1]

Analytical techniques are generally divided into two prevalent areas in the specific detection and separation sciences.

- **Specific Detection [1,2,3]**

Specific detection is an analytical determination based on specific responses related to the chemical characteristics of a molecule excited by a certain type of irradiation.

- UV - Visible spectrophotometry
- NMR spectroscopy
- Mass spectroscopy
- Atomic absorption spectroscopy
- X- ray diffraction
- Fourier transform infrared spectroscopy (FTIR)
- Attenuated total reflectance (ATR)

For the present study UV-Visible spectroscopy was selected. UV absorptions are mainly electronic in nature and are associated with resonating structures in the molecule. UV absorption is an essential tool for qualitative and quantitative determination of a single component drug or isolated extract. The UV quantitative determination, generally performed in solution, is based on the Beer–Lambert law. This law indicates that the absorbance of the solution of a molecule at its maximum wavelength is proportional to the length of the light path of a cell and the concentration of the solution.

Quantitative determination of a single drug substance in a nonchromophoric solvent such as an aqueous or alcohol solution may be a good application for the UV method. The assay of an absorbing susbstance may be quickly carried out by preparing a solution in a transparent solvent and measuring its absorbance at a suitable wavelength.

The use of A (1%, 1 cm) values avoids the need to prepare a standard solution of the reference substance in order to determine its absorptivity.

The choice of solvent is governed by the solubility of the absorbing substance and by the absorption of the solvent at analytical wavelength. In spectrophotometer the wavelength of maximum absorbance is selected by wavelength scan, the wavelength at which the maximum absorbance was attained when scanned in the range of 200-400 nm was selected as detection wavelength for the method.

- **Separation Sciences [1]**

Over the last 20 years, separation technology for analytical testing in the pharmaceutical industry has undergone great advances. This progress was motivated by the need for better quality products, the desire for improved knowledge in product development and in part, regulatory requirements. Separation techniques such as TLC, HPLC, GC, LC, counter current extraction (CCE), and capillary electrophoresis (CE).

- **HPLC (High Performance Liquid Chromatography)[1,4]**

HPLC is a physical separation techniques conducted in the liquid phase in which a sample is separated into its constituent components (or analytes) by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column). An online detector monitors the concentration of each separated component in the column effluent and generates a chromatogram. HPLC is the most widely used analytical technique for the quantitative analysis of pharmaceuticals, biomolecules, polymers and other organic compounds. HPLC provides reliable quantitative precision and accuracy, along with a linear dynamic range sufficient to allow for the determination of the API and related substances in the same run using a variety of detectors and can be performed on fully automated instrumentation. Major modes of HPLC include reverse phase and normal phase for the analysis of small (<2000 Da) organic molecules, ion exchange chromatography for the analysis of ions, size exclusion

chromatography for the separation of polymers and chiral HPLC for the determination of enantiomeric purity. Numerous chemically different columns are available within each broad classification, to further aid method development.

Reverse Phase chromatography [1, 4]

In reverse-phase HPLC, retention is based on distribution between a nonpolar stationary phase and a polar mobile phase (typically a mixture of water and acetonitrile or methanol) and elution is promoted by addition of the less polar solvent to the mobile phase. With the exception of extremely polar or ionized compounds, which are not amenable to normal-phase HPLC and extremely nonpolar compounds such as certain steroids and natural products, which are not amenable to reverse-phase HPLC but both modes of HPLC are potentially applicable to APIs and related substances. However, about 75% of current HPLC analyses are performed using the reverse-phase.

Various steps involved in RP-HPLC Method Development

- Selection of solvents
- Selection of stationary phase
- Selection of mobile phase and its ratio
- Selection of detecting wavelength
- Selection of flow rate etc.

- **HPTLC (High Performance Thin Liquid Chromatography) [5]**

HPTLC is a highly useful method for both quantitative and qualitative anlaysis. It is an advanced form of thin layer chromatography. HPTLC is superior to other analytical techniques in terms of total cost and time for analysis. It is an offline process in which various stages are carried out. HPTLC has been reported to provide excellent separation, qualitative and quantitative analysis of a wide range of compounds such as herbal and botanical dietary supplements, biochemical, biological, pharmaceutical and medicinal samples.

Advantages of HPTLC

Visual chromatogram and simplicity, multiple sample handling, quantification of crude drugs, automatic sample application, simultaneous analysis of sample, several analysts works simultaneously, visual detection is possible in open system, small quantity of mobile phase is sufficient.

Various steps involved in RP-HPTLC method development

- ✓ Sample and standard preparation
- ✓ Selection of chromatographic plates
- ✓ Plates pre- washing
- ✓ Plates pre- conditioning
- ✓ Application of sample (Linomat V)
- ✓ Chromatographic development
- ✓ Detection of spots
- ✓ Scanning and documentation of chromoplate using WINCATs software

STABILITY-INDICATING METHODS [1]

Methods used to monitor the stability of pharmaceutical products must be specific to the major analyte and capable of separating the degradation and impurity peaks. The stability-indicating nature of the method can be demonstrated by subjecting the product to forced degradation (usually by heat, acid, alkali, light, and peroxide). Conditions (time and temperature) must be controlled so that no more than 20–30% degradation occurs. The degraded samples are then analyzed according to the method. For the method to be stability-indicating, the degradation peaks must be sufficiently well resolved from the major component so that the specificity and accuracy of the method are not affected.

PRESERVATIVES [6, 7]

Food is an essential thing for human survival. Expect our own garden plants, all the food used today has some preservatives. Preservatives are the substances, which are used to prevent food spoilage from microorganism. Food preservation is used from the ancient times. This will inhibit the growth of microorganisms like bacteria and fungi.

Food preservatives becomes an essential thing nowadays, this plays an important role during food transportation. This will preserve the food for a long duration from the spoilage. Each and every packaged food items has some preservatives, without them the food has no longer survive. Food preservatives aim to preserve the appearance of food, preserve the food characteristics like odor, taste and food is preserved for a long time.

Adding substances to food for preservation, flavor or appearance is a centuries old practice. Before refrigeration, salts were used to preserve meats and fish, and sugar was added to preserve fruits. In ancient cultures sulfites were used to preserve wine and spices and colorings were used to enhance flavors of foods. The food and drug administration (FDA) maintains a list of over 3,000 ingredients in its food additive database. Many 7 of these are found in our own kitchens like salt, sugar and baking soda.

The major goal for the National Food Administration (NFA) is to ensure the safety of food. In order to achieve this, it is important to perform risk assessments of microorganisms and chemicals (contaminants and food additives) in food. The major reason for using food additives is to preserve and in different ways improve the quality of food. Food additives must be of value for the consumer or necessary for the handling and distribution of the food and they have to be toxicologically acceptable. In order to keep the intake of food additives within safe levels it is important to regulate the use of the additives in food, as well as to estimate consumption of the foods that contain the specific additive.

Food additives [8]

There are two major categories of food additives, enrichment substances and the technological additives. Enrichment substances are used to improve the nutritional value of food and have been used as a tool in reducing deficiency diseases. For example, iodine is added to salt to avoid goitre and vitamin A, D are added to margarine to avoid health effects due to deficiency of these nutrients. Technological food additives are mainly used to increase the shelf life of food (preservatives and antioxidants), or to give it a better taste (sweeteners and flavouring agents), or to change its consistency (emulsifiers and thickeners). Today we have about 300 technological food additives that are approved for use and they can be divided into sub groups.

Types of food additives

The various types of food additives are,

- Anti-oxidants
- Chelating Agents
- Colouring Agents
- Emulsifiers
- Flavours and Flavour Enhancers
- Humectants
- Anti-caking Agents
- Preservatives
- Stabilizers and Thickeners

The present study is focused on the finger print analysis and determination of butylated hydroxyanisole (BHA).

Antioxidants [8]

Antioxidants are substances that can protect materials (not only foods) against autoxidation, irrespective of the mechanism of action. More exactly, such compounds should be called oxidation inhibitors, and only those substances that

inhibit oxidation by reaction with free radicals should be called antioxidants. The free radical scavenging potential is an important method for determination of antioxidant activity. Antioxidant may also inhibit the decomposition of lipid hydroperoxide, which would otherwise form free radicals.

Analysis of Antioxidants [8]

The analysis of antioxidants consists of two subsequent operation stages, isolation of antioxidants from the substrate and purification of the extract, and quantification of antioxidants in the extract. Several methods have been standardized and can be found in the respective books of analytical standards. Antioxidants are subject to changes during food storage or heating so that it would be correct to isolate and determine not only the original antioxidant, but also potential degradation products. Only in such a way would it be possible to find whether the original content of antioxidants has not exceeded the legal limit.

METHOD VALIDATION [1, 9]

It is important to define the terms used in regulatory guidelines when discussing method validation. Validation of an analytical procedure is a process required to demonstrate that the procedure is suitable for its intended use. Almost all analytical tests require some type of validation. The amount and type of validation will depend on the test procedure. Validation is necessary before an analytical test can become a test procedure in the QC laboratory. The FDA has identified seven validation characteristics: accuracy, precision, specificity, detection limit, quantitation limit, linearity, and range. Depending on the test being validated, combinations of these characteristics need to be examined.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Method accuracy is the agreement between the difference in the measured analyte concentrations of fortified and unfortified samples, the fortification procedure is called spiking.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements from multiple sampling of the same homogeneous sample under prescribed conditions. Precision may be considered at three levels: Repeatability, intermediate precision, and reproducibility.

Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time.

Intermediate precision

Intermediate precision expresses within laboratories variations: Different days, different analysts, different equipment etc.

Reproducibility

Reproducibility expresses the precision between laboratories.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

Detection Limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Quantitation Limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of the analyte in the sample.

Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of the analyte in the sample (including these concentrations) for which it has been shown that the analytical procedure has a suitable level of precision, accuracy and linearity.

Robustness

The robustness of an analytical procedure is the measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters and provides an indication of its reliability during normal usage.

Ruggedness

The United States Pharmacopeia (USP) defines ruggedness as the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions, such as different labs, different analysts and different reagents. Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst.

Sensitivity

The sensitivity of an analytical method is equal to the slope of the calibration line in a linear system.

LITERATURE REVIEW [10-22]

1. Fanny Galimany-Rovira *et al.*, (2016) reported a development and validation of a new RP-HPLC method for the simultaneous determination of hydroquinone, kojic acid, octinoxate, avobenzone, BHA and BHT in skin-whitening cream. Separation was achieved on a Zorbax SB-Phenyl (2504.6 mm; 5 mm) column, using a mobile phase consisting of water with 0.1% acetic acid and acetonitrile, in a dual-mode gradient for 14 minutes. The column was maintained at 40°C and detection was carried out at 230 nm by using a diode-array detector. The method developed was validated in accordance with ICH guidelines.

2. Juan Ignacio Cacho *et al.*, (2016) reported a method on determination of synthetic phenolic antioxidants in edible oils using microvial insert large volume injection gas-chromatography. Quantification was carried out by the matrix-matched calibration method using carvacrol as internal standard, providing quantification limits between 0.08 and 0.10 ng/g depending on the compound. The three phenolic compounds were detected in several of the samples, BHT being the most frequently found. Recovery assays for oil samples spiked at two concentration levels, 2.5 and 10ng/g, provided recoveries in the 86–115%.

3. Md. Shahadat Hossain *et al.*, (2016) reported a method on Screening of caffeine, antioxidants and preservatives in dairy products available in bangladesh using an RP-HPLC method. The evaluation was performed using a C_{18} column (150 mm× 4.6 mm i.d., 5 µm particle size) with a gradient flow rate of acetonitrile and diluted sulfuric acid (0.002 M) as mobile phase from ratio 15: 85 to 80: 20 (%v/v) at a flow rate of 2.0 ml/min. the parameters like linearity, precision, accuracy, and robustness were studied at 265 nm. The result of analysis has been validated statistically.

4. Mannan Hajimahmoodi *et al.*, (2015) reported a Gas chromatographic determination of synthetic antioxidants in liquid frying oil samples. The recoveries of BHA, BHT and TBHQ were 95.44-105.04, 96.03-105.47 and 95.62–108.89%, respectively. Fifty three commercial liquid frying oils were analyzed using the chromatographic method. The contents ofBHA, BHT and TBHQ were found to be 0.00, 0.00 and 40.18±0.21 mg/kg respectively. The levels of synthetic antioxidants in studied samples were all below the legal limits of Iran (200mg/kg).

5. Hitesh Katariya *et al.*, (2013) reported Development and Validation of UV Spectrophotometric Method for Determination of isradipine Loaded into Solid Lipid Nanoparticles. A wavelength maximum of isradipine (in Methanol: chloroform mixture) was selected at 327 nm. Method was found to be linear in the range of 5µg/mL to 30µg/mL with a correlation coefficient (r) of 0.999. This sensitive method was capable to recover accurately and precisely from 80 % level to 120 % level isradipine loaded into solid lipid nanoparticles.

6. S.Vijaya Saradhi1 *et al.*, (2013) reported a method on spectroscopic method for determination of butylated hydroxyanisole (BHA). This method was based on the formation of colored species on binding of ferrous ions with MBTH in the presence of HCl, and the colored chromogen obtained was finally treated with the antioxidant BHA to produce green color with λ_{max} at 625 nm. Statistical analysis of this method exhibited Sandal's Sensitivity of 0.0264, and the relative standard deviation (RSD) of this method was found equal to 0.8733, indicating that the developed method was reproducible, for the determination of BHA in formulations and bulk dosage forms.

7. Mohammad Younus *et al.*, (2013) reported a RP- HPLC method development and validation for quantification of isradipine in bulk and formulation. The estimation was carried out on Agilent Zorbax C_8

(4.6x150 mm, 5μ), using mobile phase consisting of methanol: acetonitrile: 0.1% OPA 55:35:10. The flow rate was 1.0 ml/min column effluents were monitored at 264 nm. The proposed method has been validated as per ICH guidelines. The retention time was 4.108 minute and the proposed method was linear in the concentration range of 10 to 150 μg/ml with coefficient of correlation 0.9998. The % recoveries at 50%, 100% and 150% were found to be 99.59, 99.49 and 99.64 respectively. All the validation parameters were within the acceptance range.

8. G. Laxmi Aswini *et al*., (2012) reported development and validation of isradipine in bulk and in its formulation by RP-HPLC. Chromatographic separation of drug was performed with kromasil C_{18} column and the mobile phase consisting of a mixture of water, methanol and tetrahydrofuran (50: 40: 10 %v/v/v), isocratic elution at a flow rate of 1.4ml/min with UV detection at 330nm at 25°C is used in this method. The proposed RP-HPLC method is successfully applied for the determination of isradipine in pharamaceutcical dosage form.

9. K.Krishna Chaitanya *et al*., (2012) reported Isocratic-Reverse Phase Liquid Chromatographic method for the quantification of isradipine by UV detection in tablets. Separation was achieved by using kromasil C_{18} (100 x 4.6mm, 5µm) column with flow rate of 1.7ml/minute and Analytes were monitored by UV detection at 326 nm, using a mixture of mobile phase containing 500 ml of water, 400ml of methanol and 100ml of tetra hydro furan mix well and sonicate to degas it. The retention time for isradipine was found to be 8.95 minutes. Calibrate curves for isradipine was linear over the concentration range 50-400 μg/ml with correlation coefficient 0.999. The percentage estimations of the isradipine in market formulations by RP-HPLC were found in between 99.61-99.86%. The recovery of the drug by standard addition method was found in range of 99.99-101% LOD and LOQ were 0.3μg/ml and 0.1μg/ml respectively. Thus the proposed method was found to be accurate, precise, reproducible and specific and can be successfully applied for quantification of isradipine in pharmaceutical dosage forms for future.

10. Mohammed Akkbik *et al*., (2011) reported as Development and validation of RP-HPLC-UV/VIS method for determination of phenolic compounds in several personal care products. The method optimized and validated for the simultaneous determination of phenolic compounds, such as butylated hydroxyanisole and butylated hydroxytoluene as antioxidants, and octyl methyl cinnamate as UVB-filter in several personal care products. The concentrations of phenolic compounds in these personal care samples were below than maximum allowable concentration in personal care formulation.

11. Shiva Kant Dwivedi *et al*., (2010) reported a Gas chromatography/mass spectrometry method for determination and confirmation of BHA, BHT and TBHQ in vegetarian ready to eat meals. Analytical characteristics of the GCMS method such as limit of detection, linear range, and recovery were evaluated. By using external standard method the analytical results showed that the linear correlation coefficients of TBHQ, BHA and BHT were more than 0.998 and Recoveries (n = 6) of the synthetic phenolic antioxidants when spiked to ready to eat food at 5,10 and 30mg kg-1 were in the ranges 97.3-105.2% for BHA, 102.7-104.7% for BHT and 98.4-101.5% for TBHQ. The lowest detection limit was 0.1mg/kg for TBHQ & BHA and 0.05mg/kg for BHT. The levels of synthetic phenolic antioxidants in all food items analysed were below the legal limits.

12. Li Xiu-Qin *et al*., (2008) reported a high performance liquid chromatography time-of-flight mass spectrometry using negative ion mode for the identification and estimation of eleven preservatives and synthetic antioxidants in edible vegetable oil samples. The eleven compounds behave linearly in the 0.05–5.0 mg/kg concentration range, with correlation coefficient greater than 0.997. The recoveries at the tested concentrations of 0.1–2.0 mg/kg are 65.8–106.9%, with coefficients of variation less than 8.1%. The method reported was suitable for routine identification and estimation of preservatives and synthetic antioxidants in edible vegetable oils.

13. Hsiu-jung lin *et al*., (2003) reported a method on effects of extraction solvent on gas chromatographic quantitation of BHT and BHA in fifteen chewing gum. The results showed that among seven types of extraction solvent, diethyl ether was the best, as chewing gum dissolved and dispersed in diethyl ether, gave the highest yield of BHT and BHA from chewing gum. Recovery studies were performed on chewing gum, spiked with both BHT and BHA at 100 to 200µg/g each. The recoveries of both BHT and BHA were 99 to 101% and 94 to 99% respectively. The coefficients of variation were less than 8.6%. Both the BHT and BHA contents were found to be 0 to 296 µg/g and 0~133 µg/g, respectively.

AIM AND OBJECTIVE OF THE WORK

Literature survey reveals that various analytical methods has been developed and validated for the estimation of isradipine using RP-HPLC. Only one method has been reported on UV spectrophotometric method for the determination of isradipine in solid lipid nanoparticles. However, no method has been reported on HPTLC technique for the estimation of isradipine in bulk and its application to degradation studies.

Apart from this the presence work aim for the analysis of butylated hydroxyanisole in selected food stuffs.

Butylated hydroxyanisole is a phenolic antioxidant which is used to prevent rancidity of fats and oils in food by protecting against oxidation. When the food additives amendment was enacted (1958), BHA were listed as common preservative and considered generally recognized as safe (GRAS). GRAS regulations limit BHA to 0.02% or 200ppm of the fat or oil content of the food products. Based on animal studies, the national toxicology program has concluded that BHA is reasonably anticipated to be a human carcinogen. Hence this project aims at quantifying of BHA present in locally available various food products to check whether the BHA are within the acceptable limit given in FDA using modern analytical techniques like RP-HPLC and RP-HPTLC.

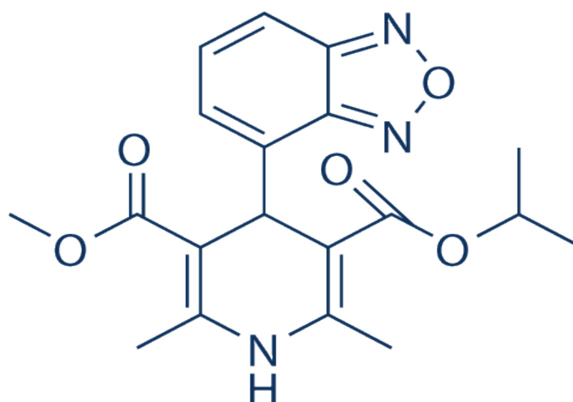
Hence the objective of the work,

Development of validated UV spectroscopic method and stability indicating HPTLC method for the estimation of isradipine in bulk drug.

Development of validated of RP- HPLC and RP- HPTLC method for the estimation of butylated hydroxyanisole in various food products.

DRUG PROFILE [27, 28]

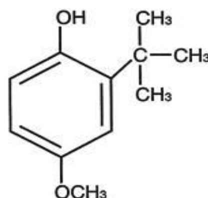
Name	: Isradipine
Structure	:



Molecular formula	:	$C_{19}H_{21}N_3O_5$
IUPAC	:	3-methyl 5-propan-2-yl 4-(2,1,3-benzoxadiazol-4-yl)-2,6- dimethyl-1,4-dihydropyridine-3,5-dicarboxylate
Molecular weight	:	371.393 g/mol
Category	:	Cardio vascular agent
λ max	:	326nm
Solubility	:	Soluble in methanol
Brand names	:	Dynacirc, Dynacirc CR

BHA PROFILE [23-26]

Structure:



Name	:	Butylated hydroxyanisole
Preservative code	:	E320
Molecular formula	:	$C_{11}H_{16}O_2$
Molecular weight	:	180.24
IUPAC name	:	2-tert-Butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole
Description	:	White or yellowish waxy solid
Melting point	:	58- 70° C
Solubility	:	Soluble in propylene glycol, ethanol, methanol and ether. Insoluble in water.
Category	:	Antioxidants and preservative. It is used in a wide range of cosmetics, foods and pharmaceuticals. When used in food products, it delays oxidative rancidity of fats and oils, and prevents loss of activity of oil- soluble vitamins. It may be found in pharmaceutical gels, creams and liquid or gelatin capsules, tablets and other pharmaceutical dosage forms.
Safety limit of BHA	:	FDA recommended 0.02% or 200 ppm

MATERIALS AND INSTRUMENTS

a) Pure drug sample

- Butylated hydroxyanisole were supplied from Himedia Laboratories Pvt, Ltd., Mumbai, India.

b) Chemicals and solvents used

- Hydrochloric acid - LR grade
- Hydrogen Peroxide - AR grade
- Toluene - AR grade
- n- hexane - AR grade
- Methanol - AR grade
- Glacial acetic acid - AR grade
- Acetonitrile - HPLC grade
- Water - HPLC grade
- Sodium Hydroxide
- Distilled water

All the above chemicals and solvents were supplied by S. D. Fine Chemicals Ltd., India and Qualigens Fine Chemicals Ltd., Mumbai, India.

c) Materials used

- Pre- coated silica gel $60F_{254}$ on aluminium sheets were procured from Merck, Germany.
- Pre-coated silica gel RP-18 F_{254} aluminium sheets were procured from Merck, Germany.

d) **Instruments used**

- Shimadzu Digital Electronics Balance
- Jasco V-630 Spectrophotometer
- CAMAG HPTLC System (with TLC Scanner, WinCATs software and Linomat V as application device)
- Shimadzu HPLC system with SPD-M10 A VP system PDA with 20µl fixed volume manual injector and LC-MS software.
- Hot air oven (Inlab Equipment Madras Ltd.)

DEVELOPMENT OF VALIDATED UV SPECTROSCOPIC METHOD FOR THE ESTIMATION OF ISRADIPINE IN BULK DRUG

Selection of solvent

The solubility of isradipine was checked with different solvent like methanol, ethanol, acetone and acetonitrile. The solvent which gave good stability and good spectrum was selected as solvent of choice for the determination of isradipine.

Preparation of stock solutions

A stock solution of isradipine was prepared by dissolving 10 mg of drug in 10 ml of methanol to get a concentration of 1000µg/ml. From the stock 5ml was further diluted to 10ml to get a concentration of 500µg/ml.

Preparation of working standard

From the stock solution containing 500µg/ml of isradipine about 0.1 to 1ml were transferred into 10ml standard flasks and made upto the volume using methanol to get a concentration ranging from 5 - 50µg/ml.

Selection of wavelength

The stock solution was suitably diluted with methanol so as to contain 10µg/ml of isradipine. This solution was scanned in the UV region and found that isradipine exhibited maximum absorbance at 326nm. Hence 326 nm was selected for the proposed study.

VALIDATION OF THE METHOD

The developed method was validated in terms of parameters like linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), robustness and stability studies.

Linearity and range

From the working stock solution containing 500µg/ml of isradipine series of concentrations from 5- 50µg/ml was prepared using methanol. The absorbance of standards were measured. The calibration graph was plotted between concentration versus absorbance. The slope, intercept, and correlation coefficient values were calculated.

Precision

Precision of the method was determined by repeatability measurement, intra day and inter day precision studies.

a) Repeatability

The repeatability was studied by carrying out the analysis of the standard drug concentration (20µg/ml) in the linearity range of the drug for six times on the same day and the response for each concentration was recorded and % RSD was calculated.

b) Intra–day precision

Intra day precision was studied by carrying out the analysis of the standard drug concentration (30µg/ml) for six times on the same day and % RSD was calculated.

c) Inter day precision

Inter-day precision was studied by carrying out the analysis of the standard drug concentration (30µg/ml) for two different days and % RSD was calculated.

Accuracy

Recovery study was carried out in order to ensure accuracy and reliability of the proposed method. It was done by addition of known quantities of the standard drug at 50% and 100% level with the preanalysed sample and the contents were reanalyzed by the developed method. The % recovery was calculated using the following formula,

$$\% \, recovery = \frac{(amount\ of\ drug\ found\ after\ the\ addition\ of\ the\ standard\ drug) - (amount\ of\ drug\ found\ before\ the\ addtion\ of\ std.drug)}{amount\ of\ standard\ drug\ added} \times 100$$

Stability studies

The concentration of 20µg/ml of isradipine was stored at room temperature and the absorbance of solution was measured at different time intervals.

Assay procedure for bulk drug

Isradipine was assayed by the developed UV spectrophotometric method. 10 mg of isradipine was weighed accurately and transferred to 10ml volumetric flask. It was dissolved by adding methanol and made upto the volume with the same. Pipetted out 0.2 ml of the stock solution into a 10ml volumetric flask and made upto the mark using methanol. The absorbance was measured for the resulting solution at the maximum at about 326nm. The amount of isradipine was calculated using the prepared linear graph.

DEVELOPMENT OF VALIDATED HPTLC METHOD FOR THE ESTIMATION OF ISRADIPINE IN BULK DRUG

Selection of parameters for method development

Selection of solvent

The solubility of isradipine was checked with different solvent like methanol, ethanol, acetone and acetonitrile. The solvent which gave good stability and good spectrum was selected as solvent of choice for the determination of isradipine.

Preparation of stock solutions

A stock solution of isradipine was prepared by dissolving 10 mg of drug in 10 ml of methanol to get a concentration of 1000µg/ml. From the stock 1ml was diluted to 10ml to get a concentration of 100µg/ml.

OPTIMIIZATION OF CHROMATOGRAPHIC CONDITIONS

Selection of mobile phase

A mobile phase system that would give dense compact spots and good separation from solvent front and application position was to be selected. Initially, different solvent systems such as methanol: chloroform, methanol: n-propanol, methanol: n-hexane, toluene: methanol were tried. Finally toluene: methanol was selected as mobile phase for the further study.

Effect of ratio of mobile phase

After selecting the mobile phase of toluene: methanol different ratio were tried (5: 5, 8: 2, 2: 8, and 9: 1 %v/v). From this 9: 1% v/v was taken for further analysis.

Fixed Experimental Parameters

Stationary phase	Pre-coated silica gel 60 F_{254} aluminium sheet
Mobile phase	Toluene: methanol: glacial acetic acid (9:1:0.05%v/v)
Saturation time	15 minutes
Migration distance	80mm
Bandwidth	6mm
Slit dimension	5×0.45 mm
Source of radiation	Deuterium lamp
Wavelength scanning	331nm
R_f value	0.34 (±0.03)

VALIDATION OF THE DEVELOPED HPTLC METHOD

The developed HPTLC method was validated according to ICH guidelines in terms of linearity and range, accuracy, precision, LOD, LOQ, robustness and stability.

Linearity and range

From the stock solution (100µg/ml) 1 - 6µl were applied as band on the plate in order to obtain concentration of 1 - 6µg/band. After development the plate was scanned at 331nm and peak area were noted. The calibration curve was plotted using peak area versus concentration of the standard solution. The slope, intercept, and correlation co-efficient value was calculated.

Precision

Precision of the method was determined by repeatability measurement, intra day and inter day precision studies.

a) Repeatability measurement

The repeatability measurement was assessed by spotting 3µl of standard solution on the plate for six times on the same day and the response for each concentration was recorded and % RSD was calculated.

b) Intra day precision

Intra day precision was studied by spotting 4µl of isradipine on the plate for six times on the same day and % RSD was calculated.

c) Inter day precision

Inter-day precision was studied by spotting 4µl of isradipine on the plate for six times for two different days and % RSD was calculated.

Accuracy

Recovery studies of the drug were carried out for the accuracy parameter. It was done by adding a known quantity of standard drug with the pre-analysed sample formulation and the contents were reanalysed by the proposed method. This was carried out at 50% and 100% levels. The percentage recovery and % RSD were calculated.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were calculated by using the equation,

$$LOD = 3.3 \times \sigma / S$$

$$LOQ = 10 \times \sigma / S$$

Where, σ is the standard deviation of y intercepts of regression line, & S is slope of calibration curve.

Robustness:

The robustness of the method is its ability to remain unaffectable by small changes in chromatographic parameters. Here the effect of change in conditions such as ratio of mobile phase (±0.1ml) and saturation time (±0.2min) were studied to prove robustness.

Stability of solution

The standard solution (3µg/band) of isradipine was kept under room temperature. Stability was studied by looking for any change in R_f value and peak area when compared to chromatogram of freshly prepared standard solution.

Stress studies

Forced degradation studies:

For conducting the forced degradation studies, the sample was subjected to various stress conditions like acid hydrolysis, alkaline hydrolysis, oxidative degradation, thermal degradation, and photolytic degradation. The study was conducted separately for isradipine in bulk drug.

Acid Hydrolysis:

Ten milligram of isradipine was weighed accurately and transferred into 10ml standard flasks. To this 5 ml of methanol and 5 ml of 1N hydrochloric acid was added and shaken well for 2min. This solution was refluxed for 1 hour at 80˚C. From this 1ml of the refluxed sample was withdrawn and spotted on pre-coated plate.

Alkaline Hydrolysis:

Ten milligram of isradipine was weighed accurately and transferred into 10ml standard flasks. To this 5 ml of methanol and 5 ml of 1N sodium hydroxide was added and shaken well for 2 min. This solution was refluxed for 1 hour at 80˚C. From this 1 ml of the refluxed sample was withdrawn and spotted on pre-coated plate.

Oxidative Degradation:

Ten milligram of isradipine was weighed accurately and transferred into 10ml standard flasks. To this 5 ml of methanol and 5 ml of 30% hydrogen peroxide was added and shaken well for 2min. The solution was spotted on pre-coated plate after 5 hours.

Thermal degradation:

Ten milligram of isradipine was weighed and transferred to a petridish and was placed in hot air oven at 80ºC for 5 hours. To this 10 ml of methanol was added, shaken well and the solution was spotted on pre-coated plate.

Photolytic degradation:

Ten milligram of isradipine was weighed and transferred to a petri dish and was exposed to sunlight for about 5 hours. The drug solution was prepared using methanol and the solution was spotted on pre-coated plate.

The plates were developed using fixed chromatographic condition, scanned and chromatograms were recorded.

DEVELOPMENT OF VALIDATED RP- HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC METHOD FOR THE ESTIMATION OF BUTYLATED HYDROXYANISOLE IN SELECTED FOOD STUFFS

Selection and pre-washing of plate

A pre-coated silica gel RP-18 F_{254} aluminium sheet was selected for the study. Pre-washing of the plate was done with methanol and then it was activated by keeping in an oven at 70°C for 10minutes.

Selection of solvents

Ideal properties of the solvents are listed below

- Substance should be soluble in the solvent used.
- Substance should show stability in the solvent used.
- Solvent should be volatile.

Accordingly acetonitrile was selected as a solvent of choice for further studies.

Preparation of stock solution

Ten milligram of butylated hydroxyanisole was weighed and transferred to 10 ml volumetric flask and made upto the mark using acetonitrile to get a concentration of 1000µg/ml.

OPTIMIIZATION OF CHROMATOGRAPHIC CONDITIONS

Selection of mobile phase

A mobile phase system that would give dense compact spots and good separation from solvent front and application position was to be selected. Initially, different solvent systems such as petroleum ether: benzene: acetic acid, petroleum ether: benzene: acetic acid: dimethyl formamide, methanol: water, glacial acetic acid: acetonitrile: water were tried. Finally glacial acetic acid: acetonitrile: water was selected as mobile phase for the further analysis.

Effect of ratio of mobile phase

After selecting the mobile phase (glacial acetic acid: acetonitrile: water) different ratio were tried (4: 4: 2, 4: 5: 1, 4: 5: 0.5, 5: 4: 1 and 5:4: 0.5 %v/v/v). From this 5: 4: 1%v/v/v was taken for further analysis.

Fixed Experimental Parameters

Stationary phase	Pre-coated silica gel RP-18 F_{254} aluminium sheet
Mobile phase	Acetic acid: Acetonitrile: Water (5:4:1)
Saturation time	15 minutes
Migration distance	80mm
Bandwidth	6mm
Slit dimension	5×0.45 mm
Source of radiation	Deuterium lamp
Wavelength scanning	291nm
R_f value	0.7 (±0.03)

VALIDATION OF THE DEVELOPED RP-HPTLC METHOD

The developed RP-HPTLC method was validated according to ICH guidelines in terms of linearity and range, accuracy, precision, LOD, LOQ, robustness and stability.

Linearity and range

From the stock solution of BHA (1000µg/ml), 1- 3.5µl was spotted on the plate to get a concentration range of 1- 3.5 µg/band. After development the plate was scanned at 291nm and peak area were noted. The calibration curve was plotted using peak area versus concentration of the standard solution. The slope, intercept and correlation co-efficient value were calculated.

Precision

Precision of the method was determined by repeatability measurement, intra day, and inter day precision studies.

a) Repeatability measurement

The repeatability measurement was studied by spotting 2µl of BHA on the plate for six times on the same day and the response was recorded and % RSD was calculated.

b) Intra–day precision

Intra day precision was studied by spotting 2.5µl of BHA on the plate for six times on the same day and the response was recorded and % RSD was calculated.

c) Inter day precision

Inter-day precision was studied by spotting 2.5µl of BHA on the plate for six times on two different days and the response was recorded and % RSD was calculated.

Accuracy

Recovery study was carried out to find the accuracy of the method. It was done by adding a known quantity of standard drug with the pre-analysed sample and the contents were re-analysed by the proposed method. This was carried out at 50% and 100% levels. The percentage recovery and % RSD were calculated.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were calculated using the equation,

$$LOD = 3.3 \times \sigma / S$$

$$LOQ = 10 \times \sigma / S$$

Where, σ is the standard deviation of y intercepts of regression line and S is slope of calibration curve.

Robustness:

The robustness of the method is its ability to remain unaffectable by small changes in chromatographic parameters. Here the effect of change in conditions such as ratio of mobile phase (±0.1ml) and saturation time (±0.2min) were studied to prove robustness.

Stability of solution

The standard solution of BHA (2.5µg/band) was kept under room temperature. Stability was studied by looking for any change in R_f value and reduction in peak area when compared to chromatogram of freshly prepared standard solution.

Application of study to BHA in selected food stuffs

Extraction procedure

Selected food stuffs like chocos, complan, munch, kitkat, five star, dairymilk and milky bar(1gm) were weighed into series of 250ml beaker and dissolved using 10ml of acetonitrile then it was further transferred to a 100 ml separating funnel. Add about 10ml of n-hexane and shaken well for about 5min until 2 layers were separated. The mixture was allowed to stand for about 10min. The lower layer (acetonitrile) was collected. Further 2 quantities of 10 ml of acetonitrile were added to the separating funnel and extracted as above. The collected extract was filtered using whatmann filter paper and the extract was used for further analysis. From the extract 9.5ml was taken and 0.5ml standard BHA was added as internal standard, analysed under fixed chromatographic condition and from the peak area obtained the amount of BHA present in 1gm of food products was calculated.

DEVELOPMENT OF VALIDATED RP-HPLC METHOD FOR THE ESTIMATION OF BUTYLATED HYDROXYANISOLE IN SELECTED FOOD STUFFS

Selection of stationary phase

Butylated hydroxylanisole is a polar drug, hence reverse phase C_{18} column was used for the estimation of butylated hydroxyanisole.

Selection of solvent

Butylated hydroxyanisole was readily soluble and shows good stability in acetonitrile. Hence acetonitrile was selected as a solvent of choice for the estimation of butylated hydroxyanisole.

Preparation of stock solution

Ten milligram of butylated hydroxyanisole was weighed and transferred to 10ml volumetric flask and made upto the mark using acetonitrile. It was further diluted to get a concentration of 10µg/ml.

Preparation of working standard solutions

The working standard solutions of BHA was obtained by diluting the stock concentration (10µg/ml). About 0.1 ml to 1.0 ml of BHA was transferred to 10ml volumetric flask and made upto the mark using acetonitrile to obtain a concentration of 0.1 to 1.0µg/ml.

OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

Optimization of mobile phase

Optimization of mobile phase was done in order to obtain an ideal peak for butylated hydroxyanisole. The mobile phase such as acetonitrile, methanol, acetonitrile: water, methanol: water were tried. Finally acetonitrile: water was selected as mobile phase for the further study.

Effect of ratio of mobile phase

To obtain an acceptable retention time the effect of ratio on mobile phase (acetonitrile: water %v/v) with varying strength (50: 50, 20: 80, 80:20% v/v) was studied to obtain acceptable retention time. From this 80: 20% v/v was taken for further analysis.

Effect of flow rate

Keeping the mobile phase ratio 80: 20 the chromatogram was recorded at different flow rates like 0.5ml/min, 1.0ml/min and 1.5ml/min to obtain a good symmetrical peak shape and acceptable retention time. Finally 1.0ml/min was selected as flow rate for further analysis.

Fixed chromatographic conditions

Stationary Phase	:	Hibar 250- 4, 6 Lichrospher 5µm
Mobile Phase	:	Acetonitrile: water
Ratio	:	80:20
Flow rate	:	1ml/min
Operating Temperature	:	Room Temperature.
Detection wavelength	:	290nm

VALIDATION OF DEVELOPED RP- HPLC METHOD

The developed RP- HPLC method was validated according to ICH guidelines in terms of linearity and range, accuracy, precision, robustness, LOD and LOQ.

Linearity

From the working stock solution containing 10µg/ml of BHA about 0.1 to 10ml were transferred into 10ml standard flasks and made upto the volume using acetonitrile to get a concentration range of 0.1 – 1.0µg/ml. The solutions were injected into HPLC column and chromatograms were recorded at 290nm. The

calibration curve was plotted using peak area versus concentration of the standard solution. The slope, intercept, and correlation co-efficient values were calculated.

Precision

Precision of the method was determined by repeatability of injection, intra day and inter day precision studies.

a) Repeatability of injection

The repeatability was studied by carrying out the analysis of the standard drug concentration (0.5µg/ml) in the linearity range for the drug for six times on the same day and the response for each concentration was recorded and % RSD was calculated.

b) Intra–day precision

Intra day precision was studied by carrying out the analysis of the standard drug concentration (0.7µg/ml) for six times on the same day and % RSD was calculated.

c) Inter day precision

Inter-day precision was studied by carrying out the analysis of the standard drug concentration (0.7µg/ml) for two days and % RSD was calculated.

Accuracy

Recovery study was carried out to find the accuracy of the method. It was done by adding a known quantity of standard drug with the pre-analyzed sample formulation and the contents were re-analyzed by the proposed method. This was carried out at 50% and 100% levels. The percentage recovery and % RSD was calculated.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were calculated by using the equation,

$$LOD = 3.3 \times \sigma / S$$

$$LOQ = 10 \times \sigma / S$$

Where, σ is the standard deviation of y intercepts of regression line, & S is slope of calibration curve.

Robustness:

The robustness of the method is its ability to remain unaffectable by small changes in chromatographic parameters. Here the effect of change in conditions such as ratio of mobile phase (±0.1ml) and flow rate (±0.1ml) were studied to prove robustness.

Stability of solution

The standard solution of BHA (0.5µg/ml) was kept under room temperature. It was injected periodically. Stability was studied by looking for any change in retention time, resolution and peak shape by comparing the chromatogram of freshly prepared standard solution.

System suitability study

The requirements for system suitability are usually developed after method development and validation have been completed. The system suitability studies were carried out as specified in USP. These parameters include column efficiency, resolution, peak asymmetry factor, capacity factor, peak tailing factor and percentage co-efficient of variation for peak area or height of repetitive injection.

Application of the proposed method for the estimation of BHA in selected food stuffs

Extraction procedure:

Selected food stuffs like chocos, complan, munch, kitkat, five star, dairy milk and milky bar (1gm) were weighed into a series of 250ml beaker and dissolved using 10ml of acetonitrile, then it was further transferred to a 100 ml separating funnel. Add about 10ml of n-hexane and shaken well for about 5min until 2 layers were separated. The mixture was allowed to stand for about 10min. The lower layer (acetonitrile) was collected. Further 2 quantities of 10 ml of

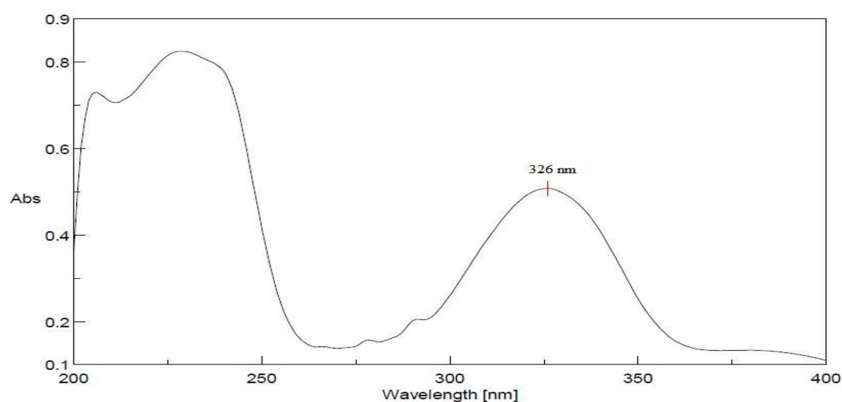
acetonitrile were added to the separating funnel and extracted as above. The collected extract was filtered using whatmann filter paper and the extract was used for further analysis. From the extract 9.5ml was taken and 0.5ml standard BHA was added as internal standard, analysed under fixed chromatographic condition and from the peak area obtained the amount of BHA present in 1gm of food products was calculated.

DEVELOPMENT OF VALIDATED UV SPECTROSCOPIC METHOD FOR THE ESTIMATION OF ISRADIPINE IN BULK DRUG

Isradipine solubility was tried with different solvent like ethanol, methanol, acetonitrile, and acetone but the drug isradipine was readily soluble in acetone and methanol. Acetone was not used due to high vaporisation. Hence methanol was selected as solvent of choice for the estimation of isradipine in bulk drug.

An ideal wavelength is one which gives maximum absorbance and good response for the drug to be detected. An UV spectrum of isradipine was recorded and shown in figure 1. From the spectrum 326nm was selected for the proposed study.

Figure 1: UV Spectrum of isradipine in methanol



The working standard solutions were prepared from the stock solution to get a concentration range from 5-50µg/ml. At 326nm the absorbance of standards were measured. The calibration graph was plotted against concentration versus absorbance. The calibration data are shown in table 1. The correlation coefficient value was found to be 0.9997. The linear graph and overlain spectrum are shown in figure 2 and 3.

Table 1: Calibration data for isradipine

Concentration (µg/ml)	Absorbance
5	0.1204
10	0.2680
15	0.3804
20	0.5076
25	0.6276
30	0.7459
35	0.8647
40	0.9835
45	1.1087
50	1.2497

Figure 2: Overlay spectrum of isradipine

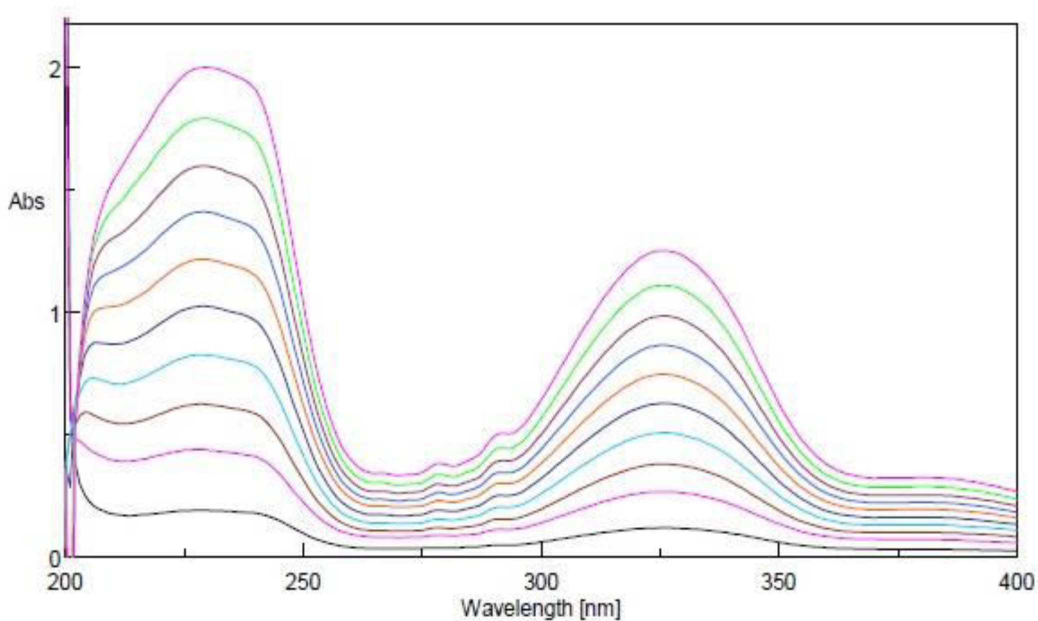
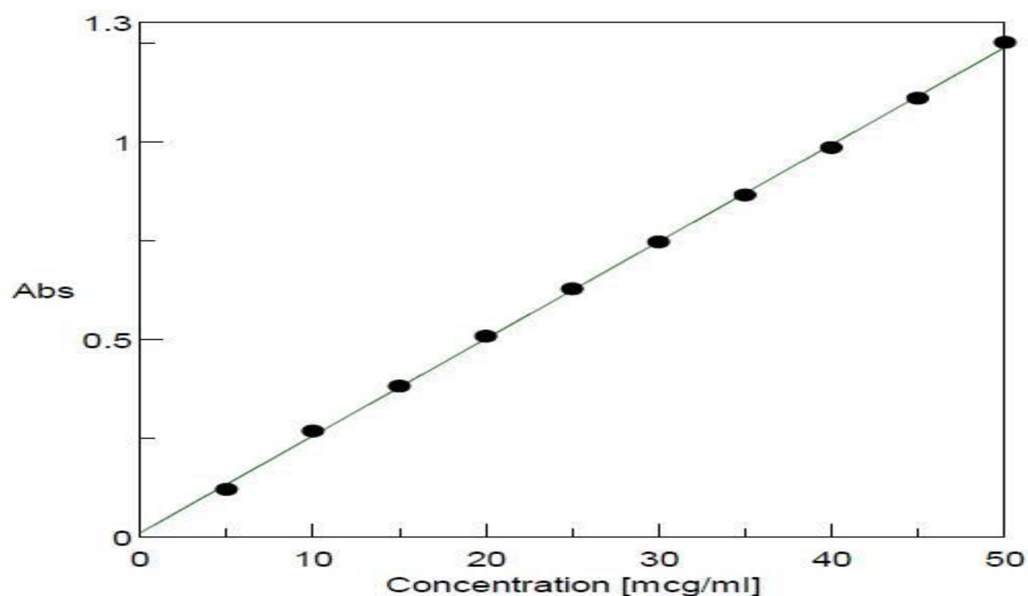


Figure 3: Linear graph for isradipine



Precision of the method was determined by repeatability measurement, intra day precision and inter day precision studies. The repeatability was studied by carrying out the analysis of the standard drug concentration (20µg/ml) in the linearity range of the drug for six times on the same day and the response for each concentration was recorded and % RSD was calculated and results are shown in table 2.

Table 2: Repeatability

Concentration (µg/ml)	Absorbance	%RSD*
20	0.5082	0.58
	0.5096	
	0.5116	
	0.5145	
	0.5147	
	0.5076	

***Mean of six determinations**

Six multiple solution of same concentration (30µg/ml) of isradipine was prepared. These solutions were measured six times and the response for each was measured. Its precision and percentage RSD were calculated and shown in table 3.

Table 3: Intra day precision

Concentration (µg/ml)	Absorbance	%RSD*
30	0.7459	0.51
	0.7387	
	0.7413	
	0.7466	
	0.7461	
	0.7491	

***Mean of six determinations**

Inter day precision was carried out by measuring a concentration (30µg/ml) of the solution for two days and the percentage RSD was calculated and given in table 4.

Table 4: Inter-day Precision

Concentration (µg/ml)	Days	Absorbance	%RSD*
30	I	0.7459	0.51
		0.7387	
		0.7413	
		0.7466	
		0.7461	
		0.7491	
	II	0.7903	1.1
		0.7679	
		0.7785	
		0.7810	
		0.7816	
		0.7941	

***Mean of six determinations**

Accuracy of the method was done by adding a known quantity of standard drug (10 and 20µg/l) with the pre- analysed sample (20µg/ml) and the contents were reanalysed by the proposed method. This was carried out at 50%, 100%, levels. The percentage recovery and % RSD was calculated and the results are shown in table 5.

Table 5: Recovery studies

Drug	Level	%Recovery	% RSD*
Isradipine	50%	102.0	0.12
	100%	101.5	0.51

***Mean of six determinations**

Stability of isradipine was checked for 20µg/ml solution which was compared using freshly prepared solution. The solution was kept at room temperature and its absorbance was measured. The drug solution was stable for about 24 hours and the values are shown in table 6.

Table 6: Stability studies

Concentration (µg/ml)	Time (hours)	Absorbance
20	0	0.5031
	24	0.5018
	30	0.4651
	48	0.3214

The absorbance was measured at 326nm for the 20µg/ml of standard solution. The amount of drug found and % RSD were calculated using the calibration graph and the results obtained for isradipine are shown in table 7.

Table 7: Result of assay of isardipine in bulk drug

Drug	Amount found (mg)	% of drug	%RSD*
Isradipine	19.96	99.87	0.05

***Mean of six determinations**

DEVELOPMENT OF VALIDATED HPTLC METHOD FOR THE ESTIMATION OF ISRADIPINE IN BULK DRUG

Isradipine solubility was tried with different solvent like ethanol, methanol, acetonitrile, and acetone but the drug isradipine was readily soluble in methanol and acetone. Acetone was not used due to high vaporisation. Hence methanol was selected as a solvent of choice for the estimation of isradipine in bulk drug.

A stock solution of isradipine was prepared by dissolving 10 mg of drug in 10 ml of methanol to get a concentration of 1000µg/ml. From the stock 1ml was diluted to 10ml to get a concentration of 100µg/ml.

Initially different solvent systems were tried and the observations are given below.

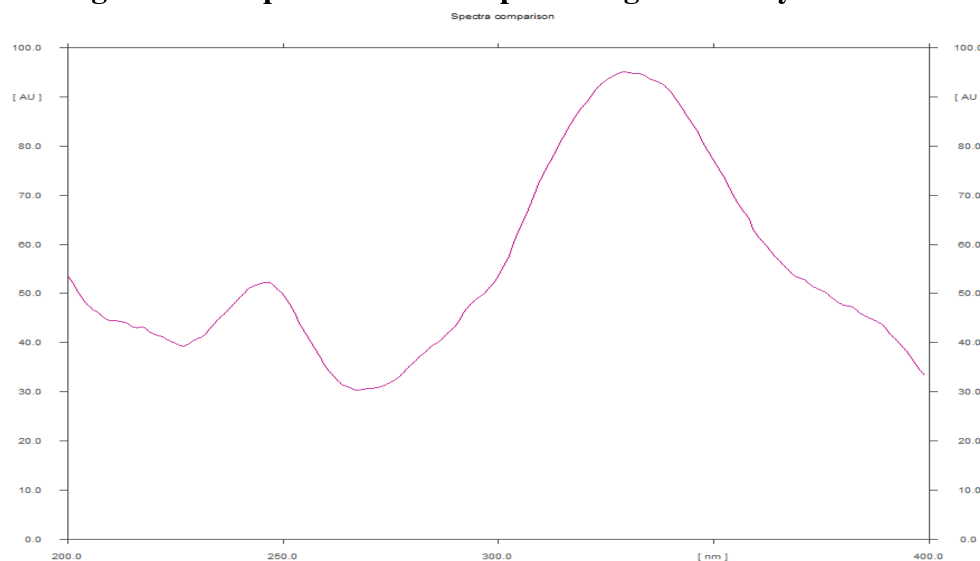
Mobile phase	Observation
Methanol: chloroform	Spot not migrated from the spot of application
Methanol : n-propanol	Spot not migrated from the spot of application
Methanol: n- hexane	Spot not migrated from the spot of application
Toluene:methanol:Glacial acetic acid	**Drug appeared as spot between spot of application and solvent front**

The mobile phase consisted of toluene: methanol in various ratio such as 5:5, 8:2, 2:8, 9:1% v/v were tried. At the ratio of 9: 1% v/v isradipine gave a dense compact spot with appropriate R_f value. Hence mobile phase with the ratio of 9: 1 %v/v was selected as an ideal ratio for the quantification of isradipine. 0.05ml of glacial acetic acid was added to the mobile phase to obtain good peak shape with acceptable R_f value.

Stationary phase	Pre-coated silica gel 60 F_{254} aluminium sheet
Mobile phase	Toluene: methanol: glacial acetic acid (9:1:0.05%v/v)
Saturation time	15 minutes
Migration distance	80mm
Bandwidth	6mm
Slit dimension	5×0.45 mm
Source of radiation	Deuterium lamp
Wavelength scanning	331nm
R_f value	0.34 (±0.01)

A spectrum of isradipine was recorded and shown in figure 4.

Figure 4: UV spectrum of isradipine using HPTLC system



The developed HPTLC method was validated according to ICH guidelines in terms of linearity and range, accuracy, precision, robustness, LOD and LOQ.

From the stock solution (100µg/ml) 1-6µl were applied as band on the plate in order to obtain concentration of 1 - 6µg/band. After development the plate was scanned and peak area was noted. The standard chromatograms are shown in figure 5-10. The peak area results are given in table 8 and the corresponding calibration graph was constructed between concentration versus peak area and shown in figure 11.

Figure 5: Chromatogram of isradipine (1µg/band)

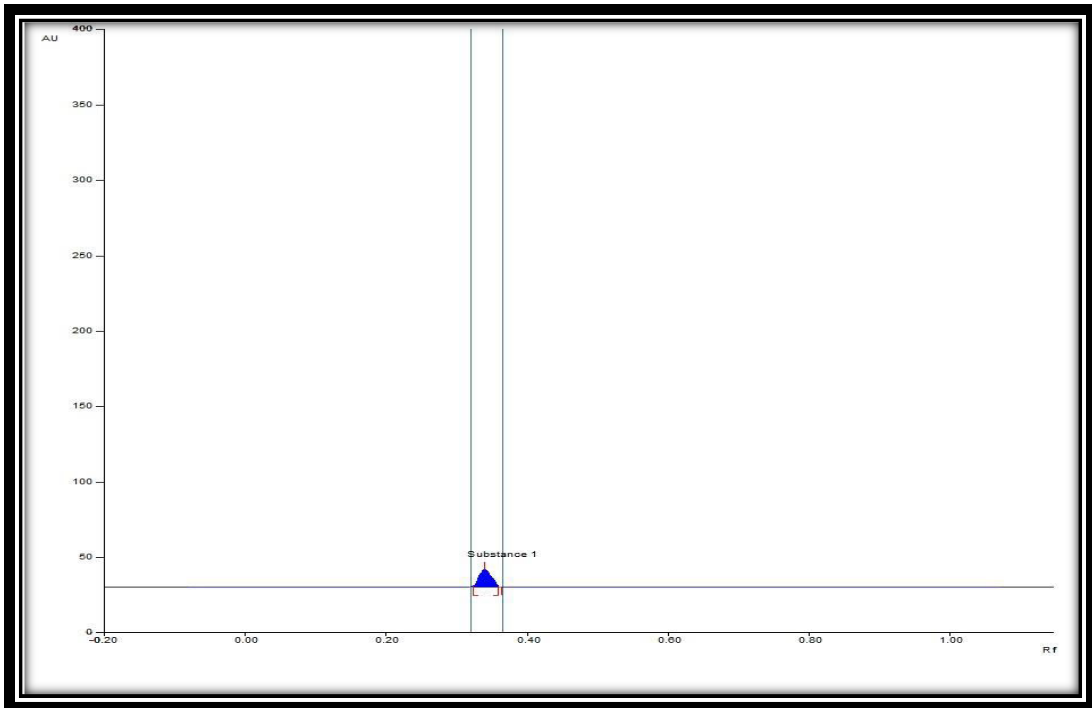


Figure 6: Chromatogram of isradipine (2µg/band)

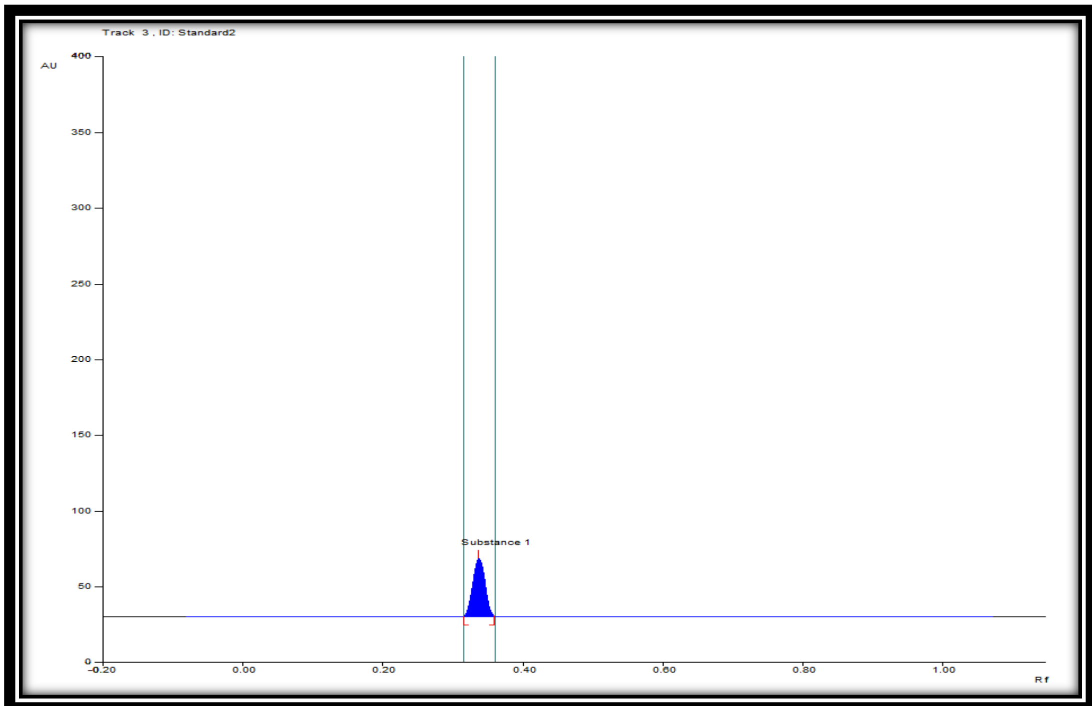


Figure 7: Chromatogram of isradipine (3μg/band)

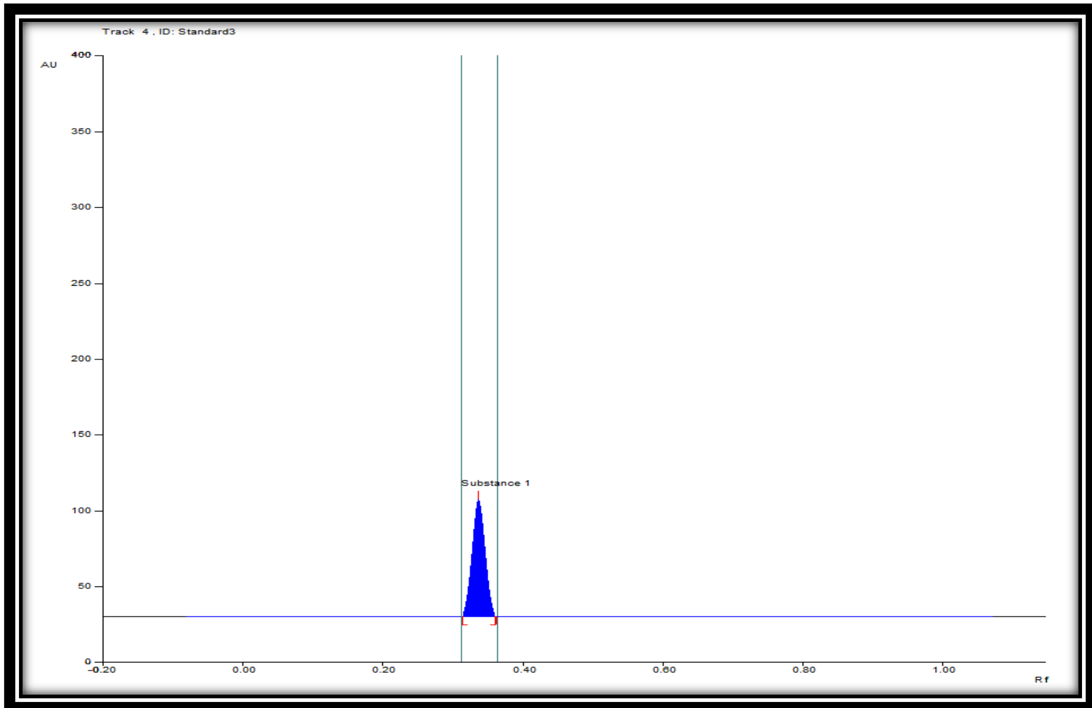


Figure 8: Chromatogram of isradipine (4μg/band)

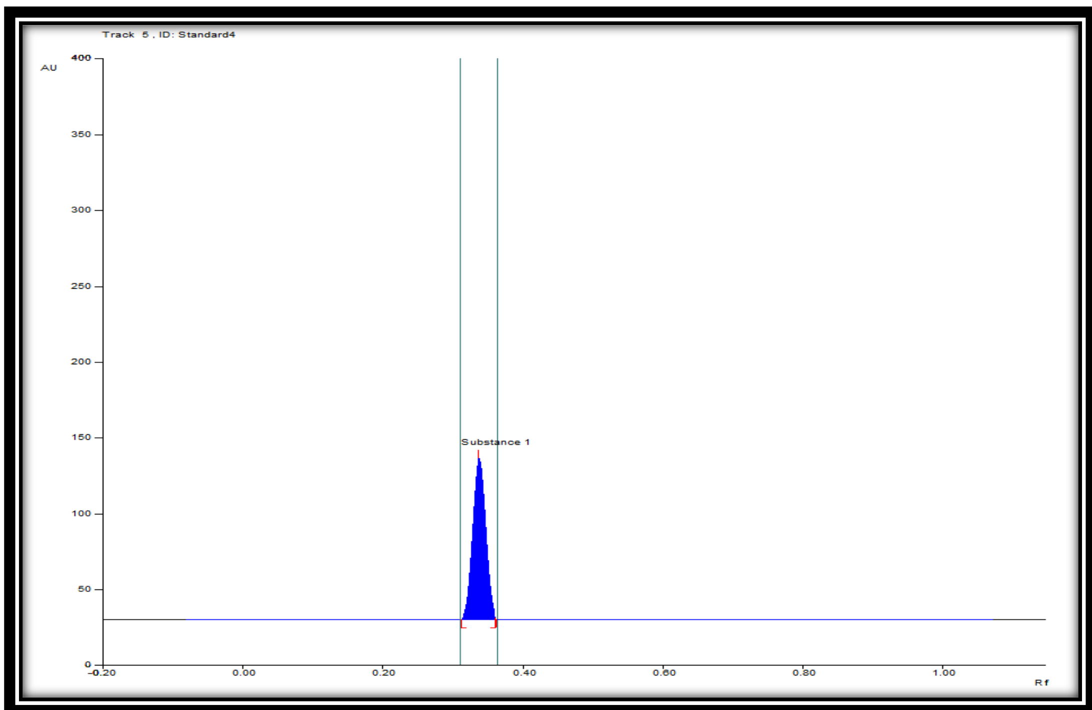


Figure 9: Chromatogram of isradipine (5µg/band)

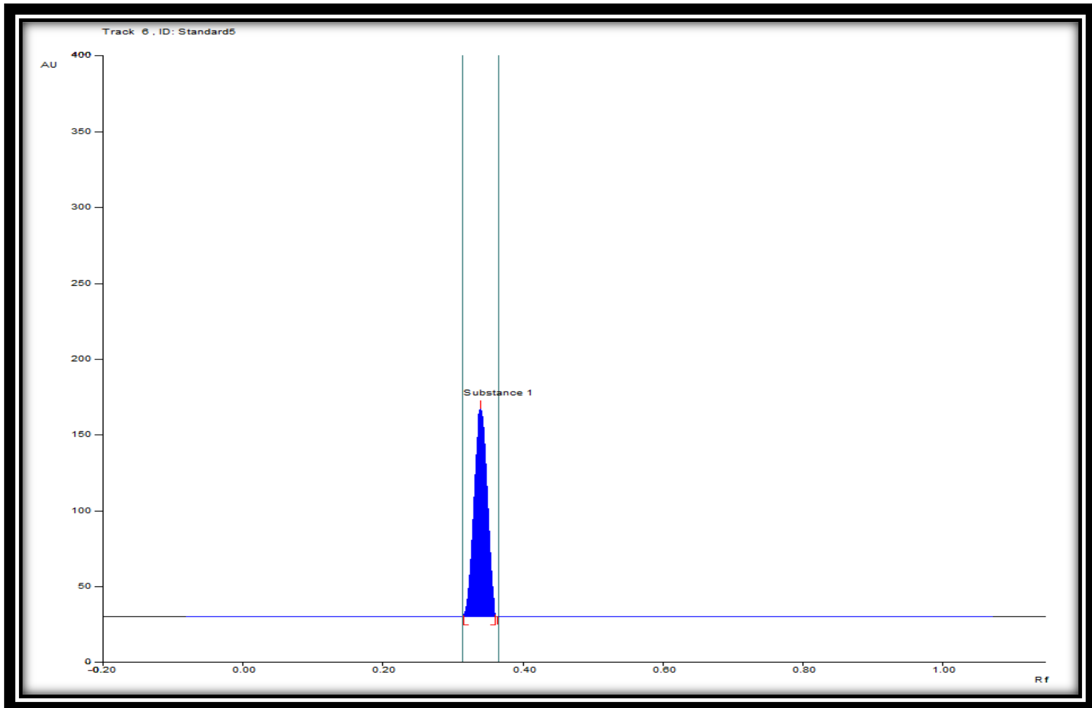


Figure 10: Chromatogram of isradipine (6µg/band)

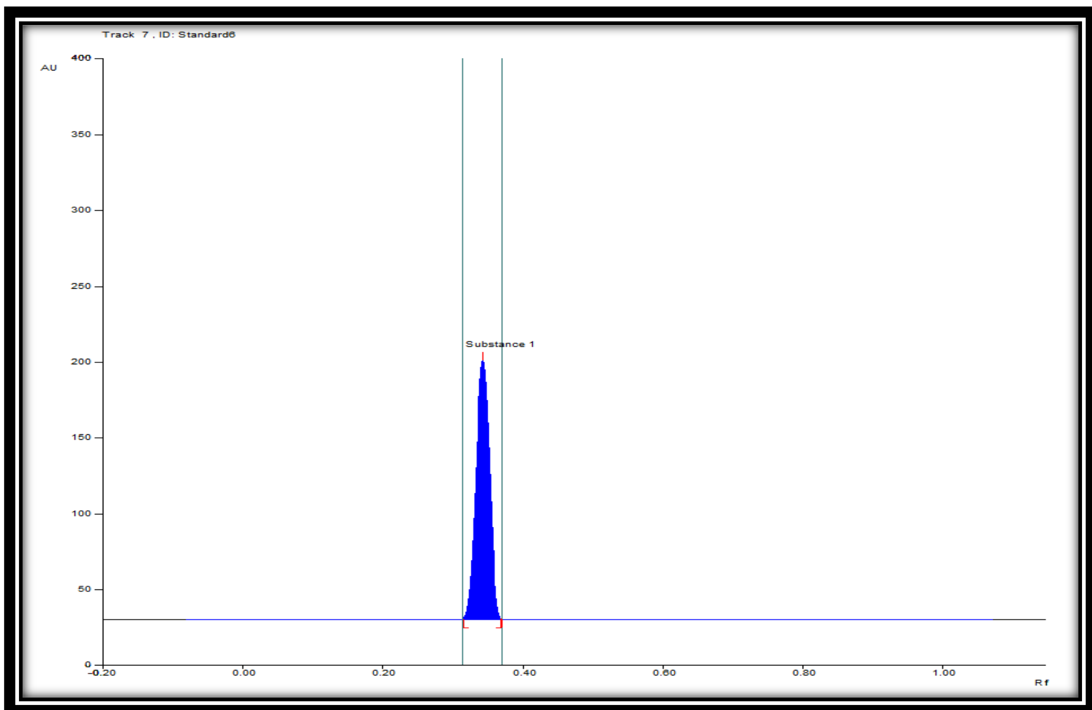
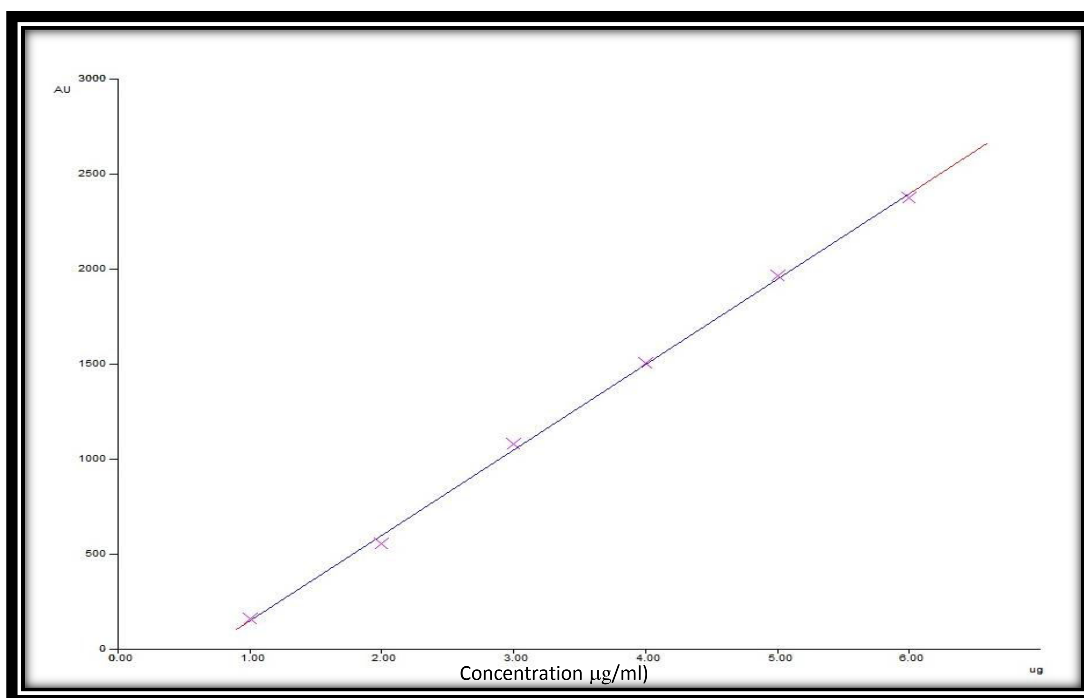


Table 8: Calibration data of isradipine

Concentration (µg/band)	Peak area
1	163.7
2	607.2
3	1087.8
4	1523.5
5	1984.1
6	2566.8

Figure 11: Calibration graph for isradipine



Precision of the method was determined by repeatability measurement, intra day and inter day precision studies.

Repeatability measurement was determined by spotting 3µl of isradipine solution on the plate for six times and the response were recorded. The percentage RSD was calculated and the results are shown in table 9.

Table 9: Repeatability measurement

Concentration (µg/band)	Peak area	%RSD*
3.0	1087.8	0.30
	1082.6	
	1086.4	
	1089.2	
	1080.9	
	1078.9	

***Mean of six determinations**

Six multiple solution of same volume 4 µl of isradipine was spotted as band six times on the plate and the response was measured. Its precision and percentage RSD were calculated and shown in table 10.

Table 10: Intra day precision

Concentration (µg/band)	Peak area	%RSD*
4.0	1525.1	1.20
	1552.5	
	1578.8	
	1547.6	
	1555.6	
	1570.7	

***Mean of six determinations**

Inter-day precision was carried out by spotting 4µl of isradipine on the plate and the response was measured. Its precision and percentage RSD were calculated and shown in table 11.

Table 11: Inter-day Precision

Concentration (µg/band)	Days	Peak area	%RSD*
4.0	I	1525.1	1.20
		1552.5	
		1578.8	
		1547.6	
		1555.6	
		1570.7	
	II	1587.9	1.60
		1593.4	
		1529.6	
		1599.3	
		1589.1	
		1560.6	

***Mean of six determinations**

To study the reliability and accuracy of the method, recovery experiments were carried out at 50%, and 100% level.

The concentration of the drug present in the resulting sample solutions were determined. The recovery procedure was repeated six times and the % recovery was calculated using the formula and results are shown in table 12.

$$\% \, recovery = \frac{(amount\ of\ drug\ found\ after\ the\ addition\ of\ the\ standard\ drug) - (amount\ of\ drug\ found\ before\ the\ addtion\ of\ std.drug)}{amount\ of\ standard\ drug\ added} \times 100$$

Table 12: Recovery studies

DRUG	% Recovery		%RSD*	
	50	100	50	100
Isradipine	101.3	101.0	0.35	0.36

***Mean of six determinations**

The LOD and LOQ were calculated by using the equation,

$$LOD = 3.3 \times \sigma / S$$

$$LOQ = 10 \times \sigma / S$$

Where, σ is the standard deviation of y intercepts of regression line, & S is slope of calibration curve. LOD was found to be 0.018 µg/band and LOQ was found to be 0.056 µg/band.

It was evaluated using a standard 0.5 µg/band, introducing a small change in the mobile phase composition, saturation time, and solvent migration distance about ± 0.1 ml for all the parameters and it was scanned under 331nm and the chromatograms was recorded. The drug isradipine showed no change in the R_f value (0.34±0.03) and the peak area. Hence the developed method found to be robust.

When the developed chromatographic plate was exposed to an atmosphere, the analyte is likely to decompose. Hence, it is necessary to conduct the stability of the plate. Stability of isradipine on the plate was studied at different time intervals and the peak area was recorded. The developed plate was stable for about 3 hour which was noted by reduction in peak area and the results are shown in table 13.

Table 13: Stability studies

Concentration (µg/ band)	Time(hours)	Peak area
3.0	0	1579.7
	1	1570.3
	2	1574.8
	3	1561.3
	4	1459.6

When isradipine was subjected to acid hydrolysis, an additional two peak were observed along with standard, R_f value of 0.34 that is shown in figure 12 and the spectrum of degradant products are shown in figure 13 and 14.

Figure 12: Chromatogram of isradipine in presence of acid degradant

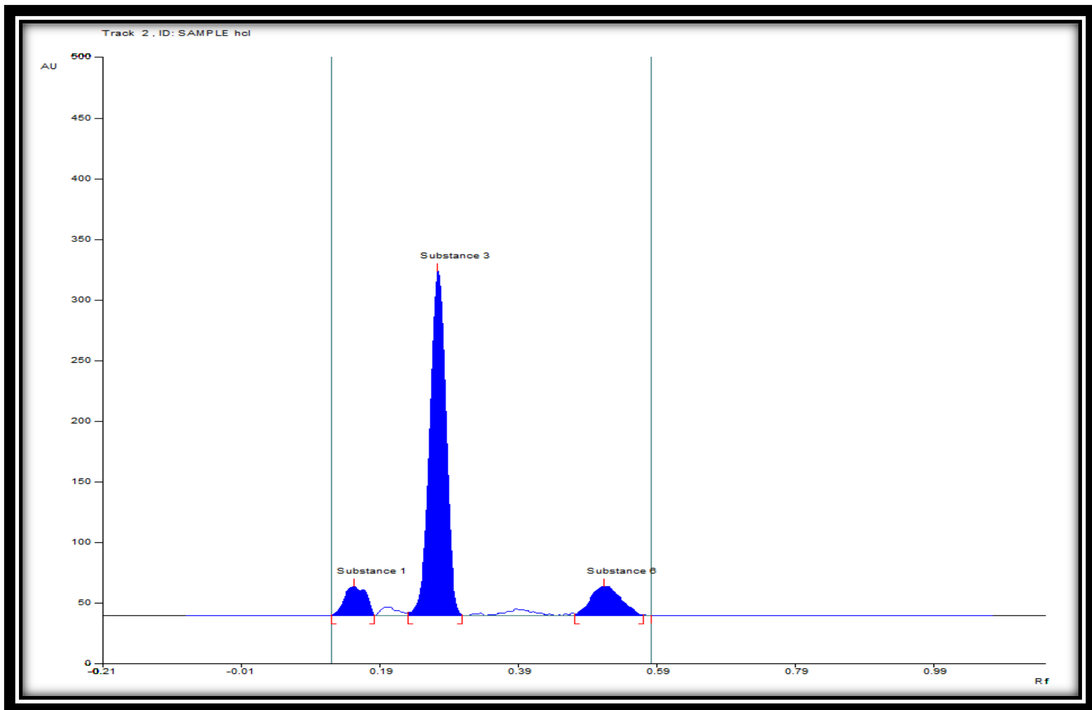


Figure 13: Spectrum of acid degradant having R_f value 0.15

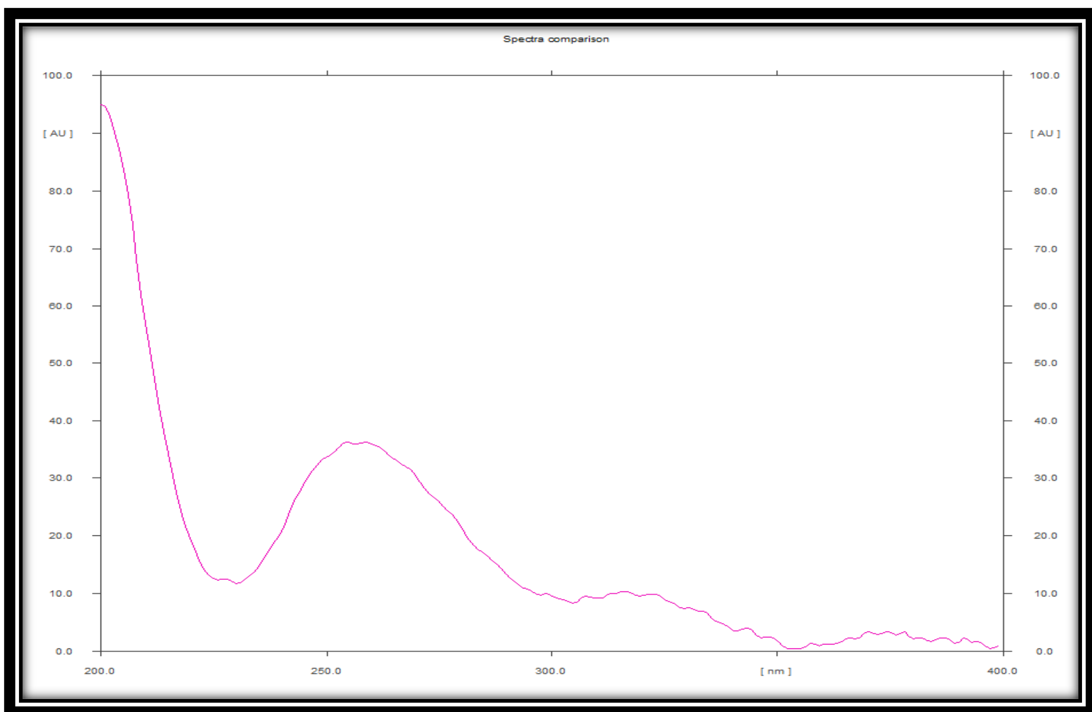
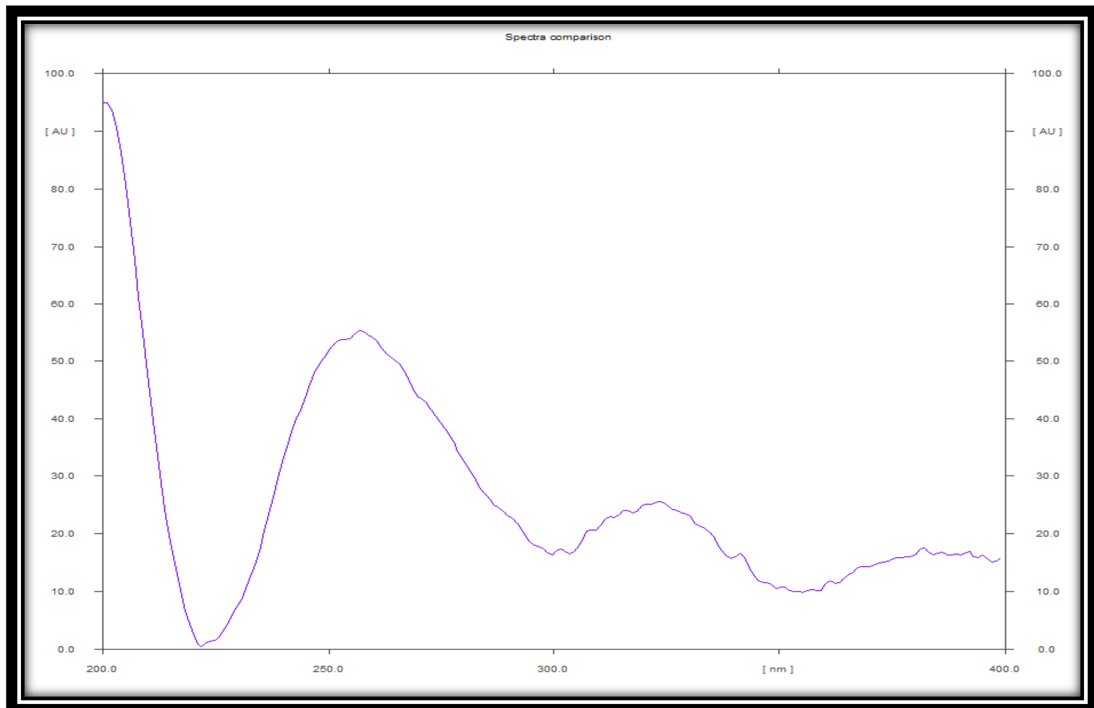


Figure 14: Spectrum of acid degradant having R_f value 0.51



Alkaline hydrolysis:

When the drug was subjected to alkaline hydrolysis additional peak were observed at R_f value of 0.20 which is shown in figure 15 and the spectrum of degradant peak are shown in figure 16.

Figure 15: Chromatogram of isradipine in presence of alkaline degradant

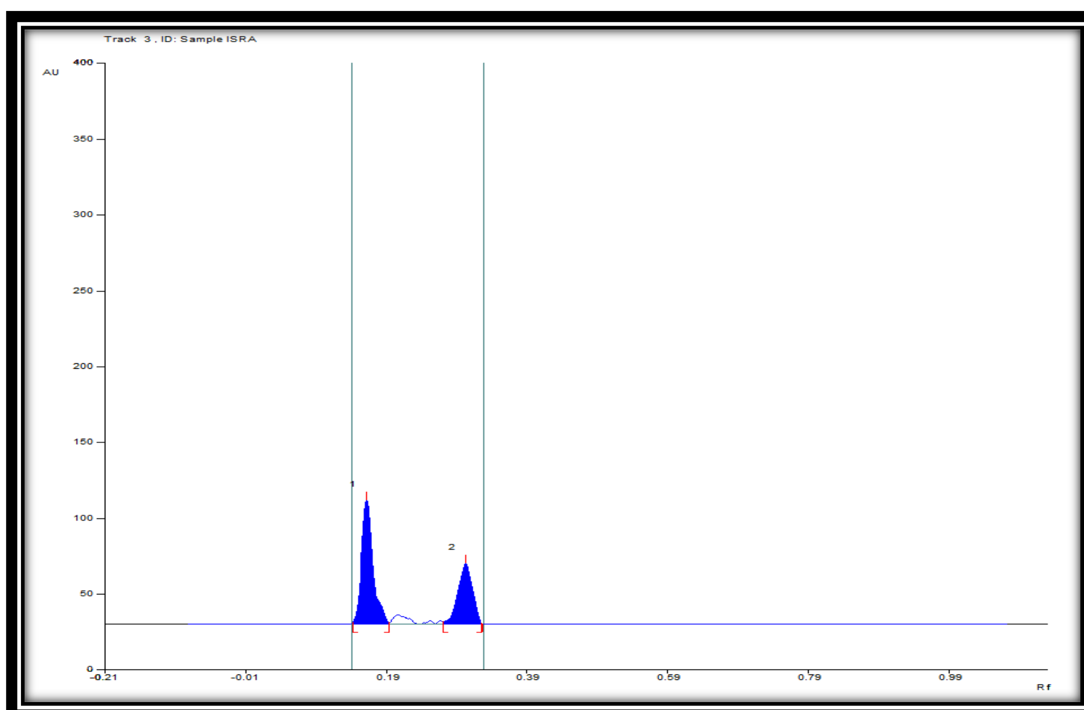
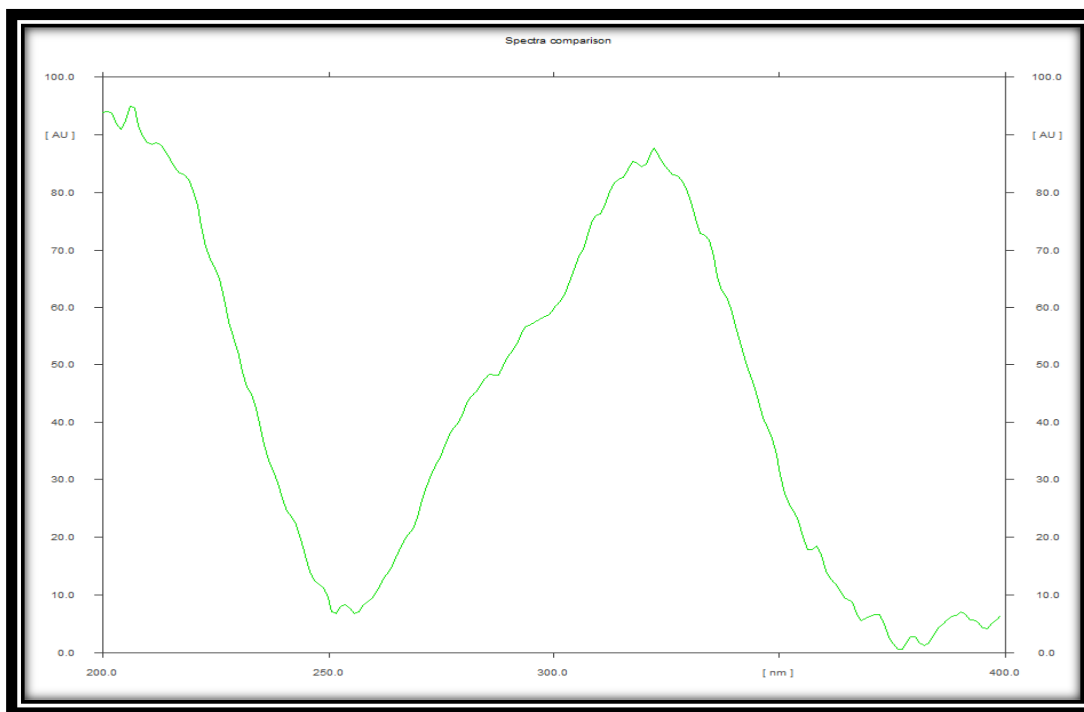


Figure 16: Spectrum of alkaline degradant having R_f value of 0.20



No additional peak was observed when standard drug of isradipine was subjected to oxidation with hydrogen peroxide, photolytic degradation and thermal degradation.

DEVELOPMENT OF VALIDATED RP-HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC METHOD FOR THE ESTIMATION OF BUTYLATED HYDROXYANISOLE IN SELECTED FOOD STUFFS

Butylated hydroxylanisole is a polar drug, hence a pre-coated silica gel RP-18 F_{254} aluminium sheet was used for its estimation. Pre-washing of the plate was done with methanol and then it was activated by keeping in an oven at 70°C for 10 minutes.

Butylated hydroxylanisole was readily soluble in methanol, and acetonitrile but it is highly stable in acetonitrile. Hence acetonitrile was selected as a solvent of choice for further study.

Initially different solvent systems were tried for the selection of mobile phase and the observations are given below in the table 14.

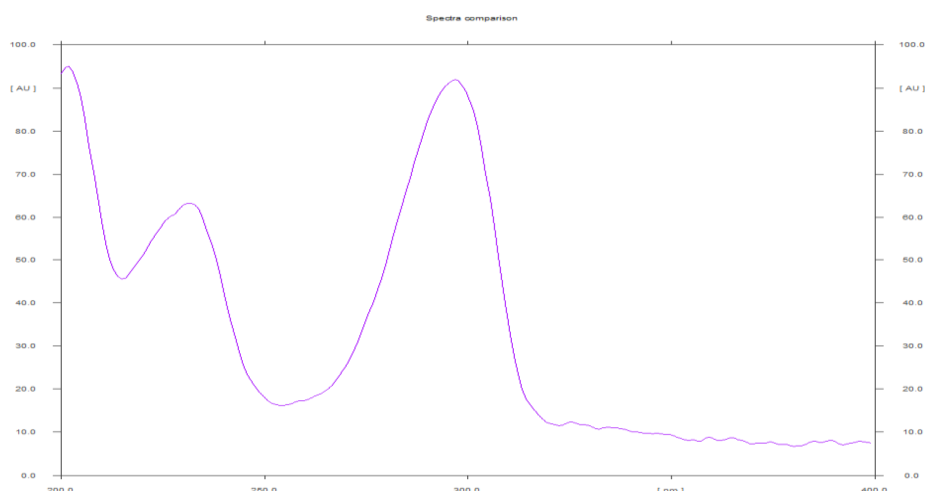
Table 14: Selection of mobile phase

Mobile phase	**Observation**
Petroleum ether : Benzene : Acetic acid	Spot not migrated from the application position
Petroleum ether: Benzene : Acetic acid : Dimethyl formamide	Spot not migrated from the application position
Methanol : Water	Spot migrated till the solvent front
Glacial Acetic acid: Methanol: Water	Spot migrated with an R_f value of 0.90
Glacial acetic acid: Acetonitrile: Water	**Compact spot with an acceptable R_f value**

Different ratios of glacial acetic acid: acetonitrile: water such as 4: 4: 2, 4: 5: 1, 4: 5: 0.5, 5: 4: 1 and 5: 4: 0.5% v/v/v were tried, from which the ratio of 5: 4: 1% v/v/v was selected as it gave dense compact spot.

The spectrum of butylated hydroaxyanisole was recorded and shown in figure 17.

Figure 17: UV spectrum of butylated hydroxyanisole using HPTLC system



After the development of RP-HPTLC method for the estimation of BHA, validation was carried out. For each parameters, % RSD was calculated.

From the stock solution of BHA (1000µg/ml), 1- 3.5 µl were applied on the plate to obtain concentration of 1-3.5µg/band. After development, the plate was scanned and peak area was noted. The standard chromatograms are shown in figure 18 - 23. The peak area results are given in table 15 and the corresponding calibration graph was constructed between concentration versus peak area and is shown in figure 24.

Figure 18: Chromatogram of butylated hydroxyanisole (1µg/ band)

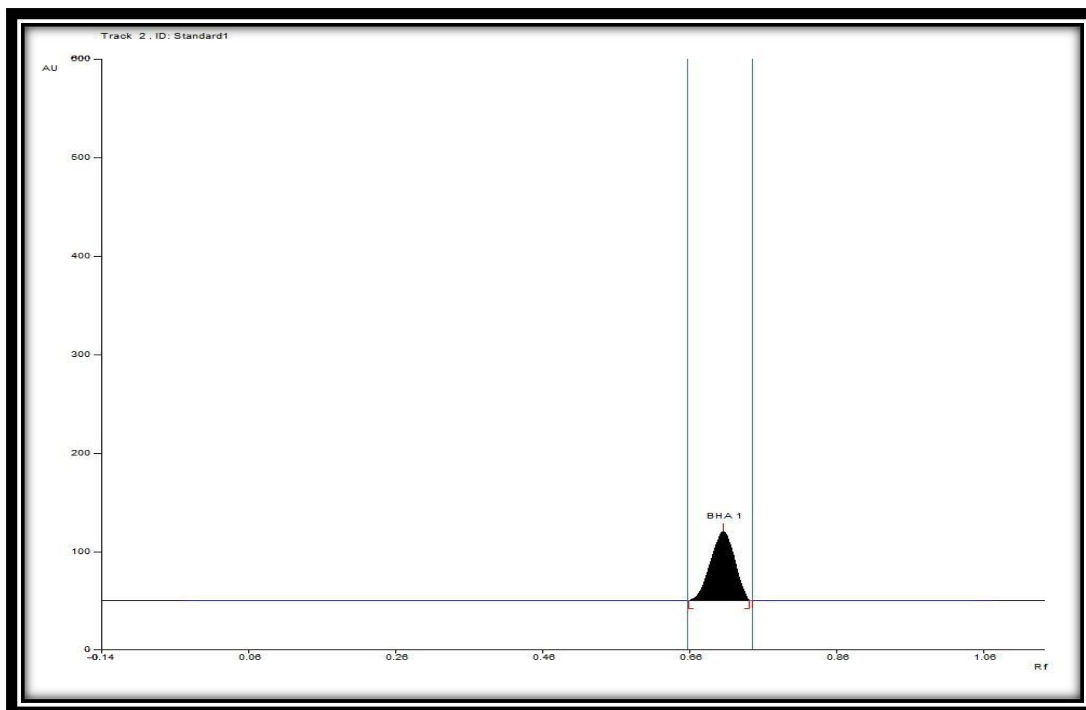


Fig 19: Chromatogram of butylated hydroxyanisole (1.5µg/ band)

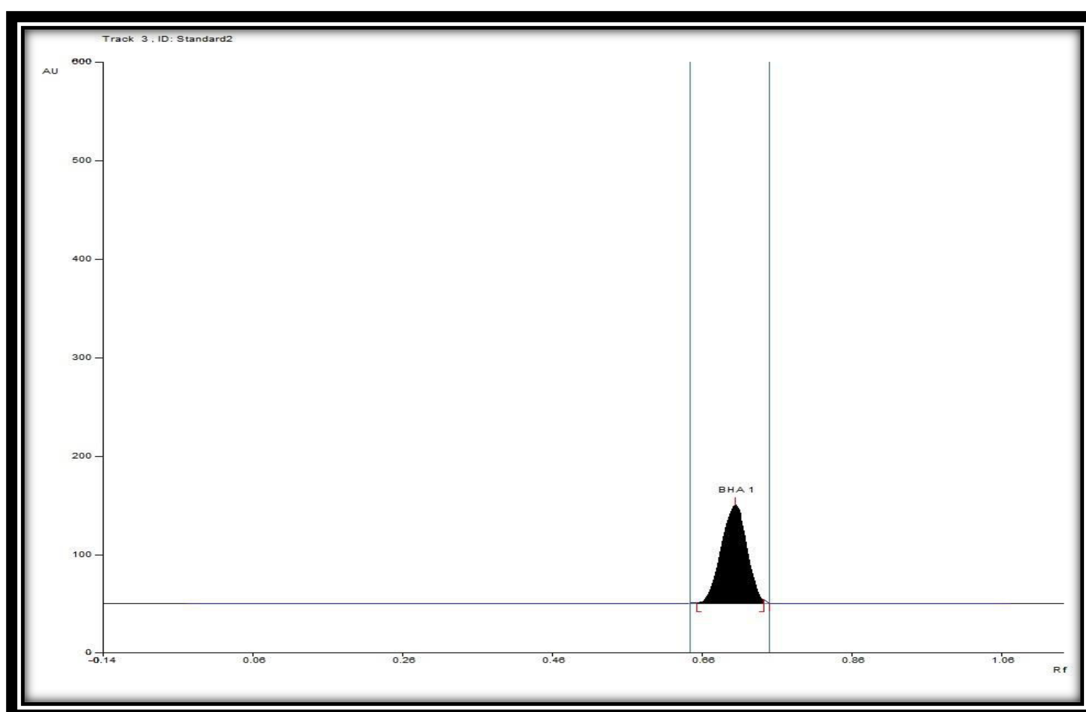


Fig 20: Chromatogram of butylated hydroxyanisole (2µg/ band)

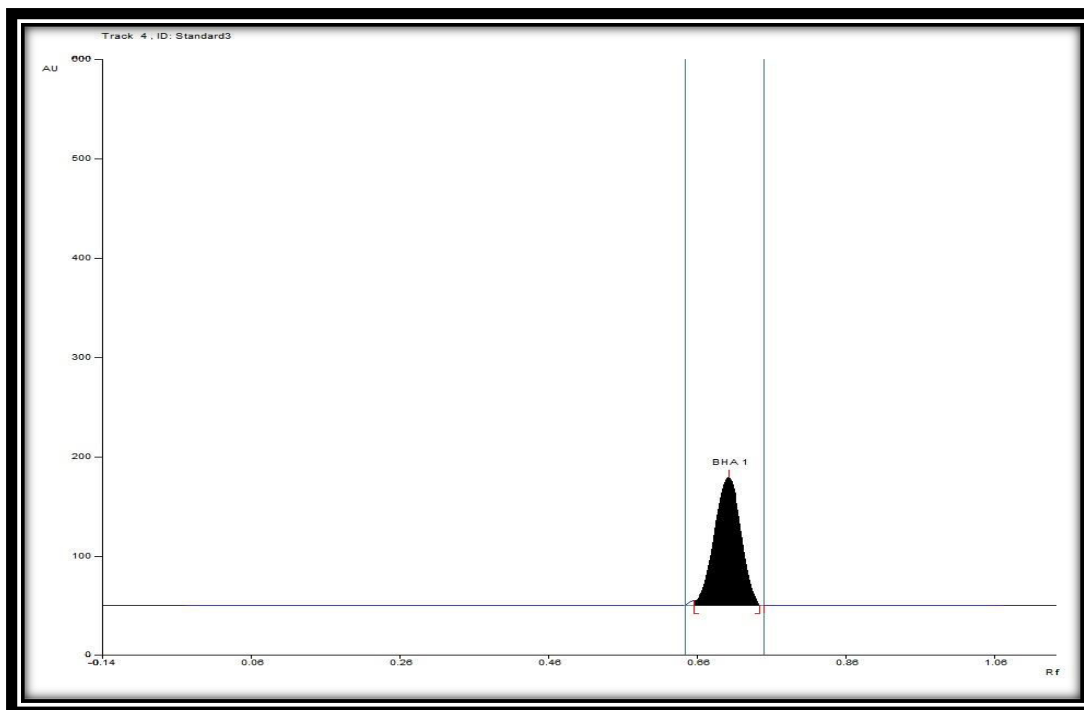


Fig 21: Chromatogram of butylated hydroxyanisole (2.5µg/ band)

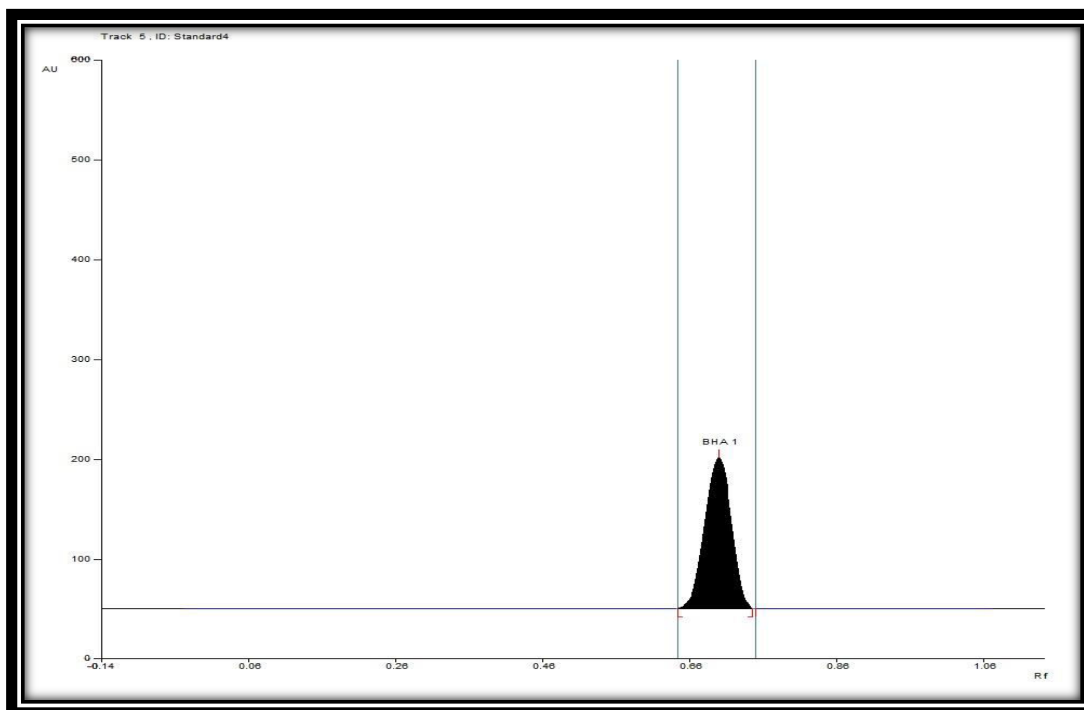


Fig 22: Chromatogram of butylated hydroxyanisole (3µg/ band)

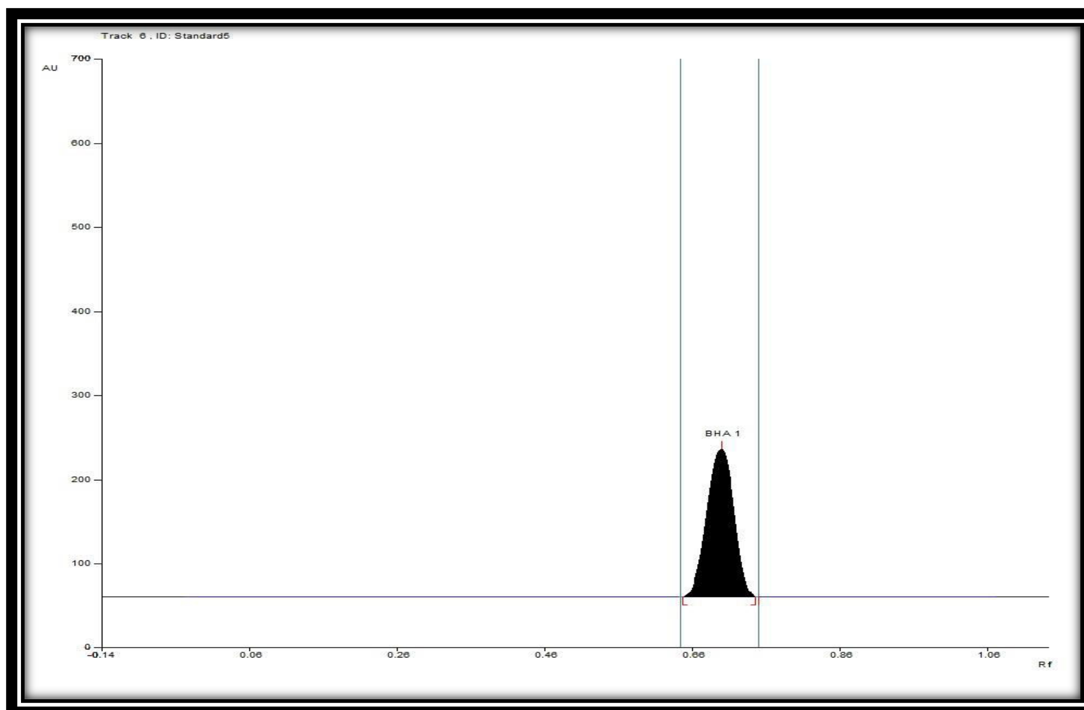


Figure 23: Chromatogram of butylated hydroxyanisole (3.5µg/ band)

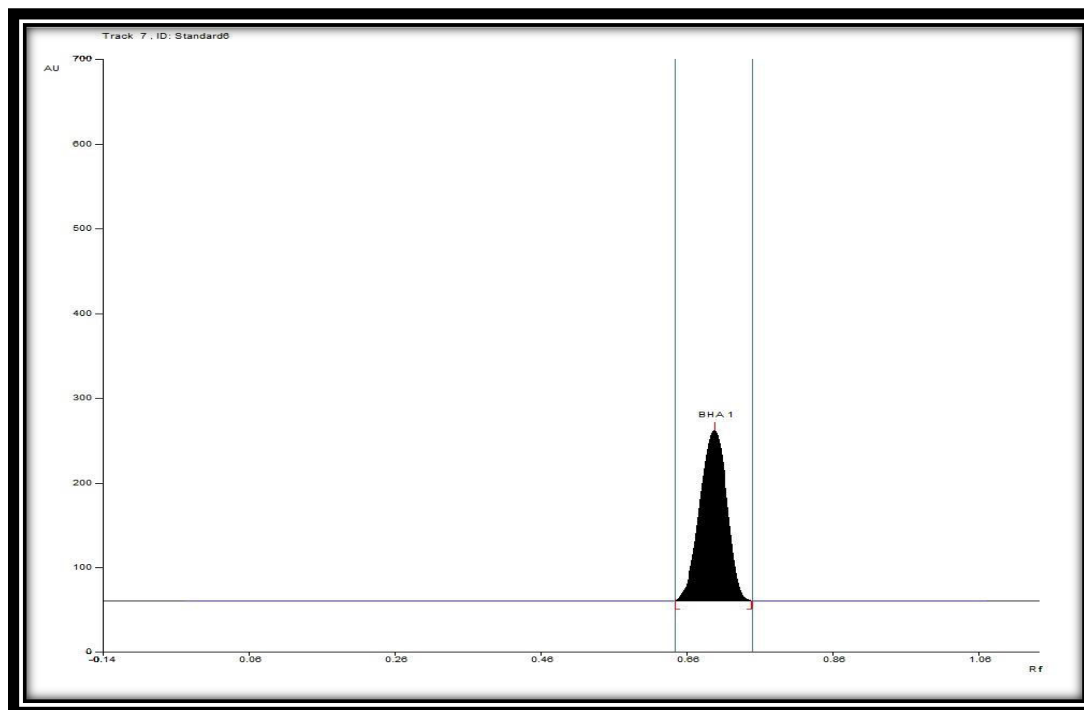
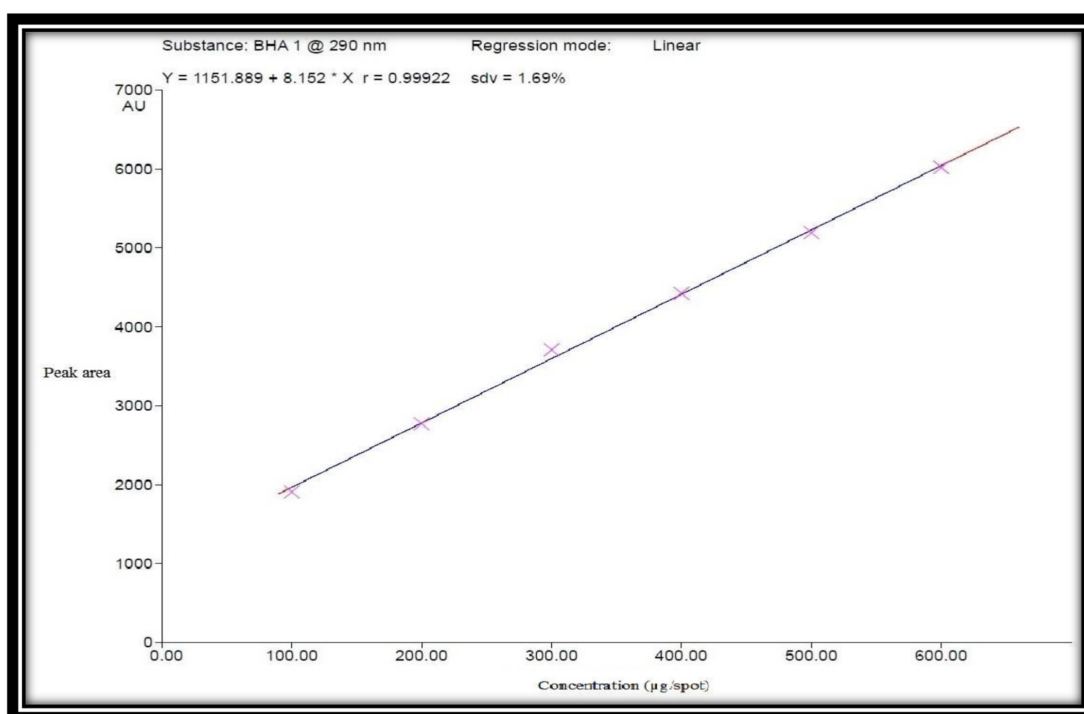


Table 15: Calibration data of Butylated hydroxyanisole

Concentration (µg/ band)	Peak area
1	1889.5
1.5	2752.2
2	3568.3
2.5	4259.2
3	5049.5
3.5	5701.3

Figure 24: Calibration graph of Butylated hydroxyanisole



Precision of the method was determined by repeatability of measurement, intra day and inter day precision studies.

From the stock concentration of 1000µg/ml, 2µl of BHA was spotted 6 times on pre-coated RP-HPTLC plate. The plate was scanned and peak area was measured. From the peak area obtained % RSD was calculated and given in table 16.

Table 16: Repeatability measurement

Concentration (µg/ band)	Peak area	%RSD*
2	3577.5	0.72
	3593.3	
	3592.7	
	3544.3	
	3529.9	
	3569.0	

***Mean of six determinations**

Intra day precision was determined by spotting 2.5µl on the plate for six times and the response for each spot was measured. The percentage RSD were calculated and is shown in table 17.

Table 17: Intra day precision

Concentration (µg/ band)	Peak area	%RSD*
2.5	4259.2	0.55
	4268.3	
	4289.8	
	4296.4	
	4230.8	
	4279.6	

***Mean of six determinations**

Inter-day precision was carried out by spotting a volume of 2.5µl of the sample solution for two days and the percentage RSD were calculated and given in table 18.

Table 18: Inter-day Precision

Concentration (µg/band)	Days	Peak area	%RSD*
2.5	I	4292.3	0.32
		4302.8	
		4265.7	
		4296.4	
		4287.3	
		4275.6	
	II	4322.6	0.31
		4309.4	
		4298.9	
		4286.3	
		4319.4	
		4306.2	

***Mean of six determinations**

This was carried out to know about the reliability and accuracy of the method. The concentration of BHA present in the 50% and 100% level were determined. The procedure was repeated six times and the % recovery was calculated using the formula and the results are shown in table 19.

$$\% \, recovery = \frac{(amount\ of\ drug\ found\ after\ the\ addition\ of\ the\ standard\ drug) - (amount\ of\ drug\ found\ before\ the\ addtion\ of\ std.drug)}{amount\ of\ standard\ drug\ added} \times 100$$

Table 19: Recovery studies

Substance	% Recovery		%RSD*	
	50	**100**	**50**	**100**
Butylated hydroxyanisole	100.28	101.17	0.23	1.30

***Mean of six determinations**

The LOD and LOQ were calculated using the equation,

$$LOD = 3.3 \times \sigma / S$$

$$LOQ = 10 \times \sigma / S$$

Where, σ is the standard deviation of y intercepts of regression line, & S is slope of calibration curve. LOD was found to be 0.03 µg/band and LOQ was found to be 0.10 µg/band.

The robustness of the method was proven by deliberate changes in conditions like mobile phase ratio (±0.1ml) and saturation time (±0.2min). The R_f value (0.70± 0.03) and peak area were found to be similar hence the developed method was found to be robust.

The standard solution of BHA (2.5µg/band) was kept under room temperature. It was spotted periodically. Stability was studied by looking for any change in R_f value and peak shape when compared to chromatogram of freshly prepared standard solution. It was observed that BHA was found to be stable for about 7 days when kept under room temperature.

Sample preparation is an important procedure in chromatographic application. One of its main aim is to remove interfering matrix components and particulates as well as to concentrate analytes of interest to enhance the sensitivity. N-hexane was selected because of its capability to remove fats and oil containing products from various food products than petroleum ether. The extracting efficiency was found to be good in acetonitrile: n-hexane compared to methanol: n-hexane. Hence acetonitrile: n-hexane was selected as the extracting solvent for BHA from other ingredients like fats, oils etc. To the extracted solution 1ml of BHA standard solution was added and spotted on the plate and scanned at 290nm the chromatograms are shown in figure 25-29. From the peak area obtained percentage BHA was calculated for each food stuffs and the results are given in table 31.

Fig 25: Chromatogram for chocos extract

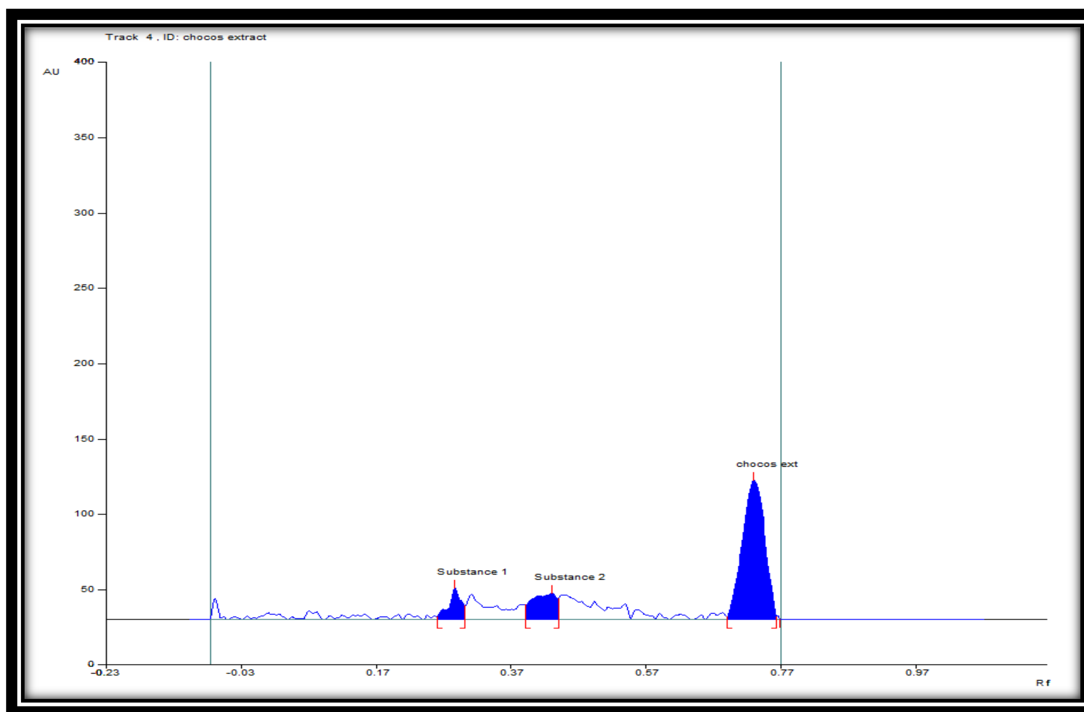


Fig 26: Chromatogram for complan extract

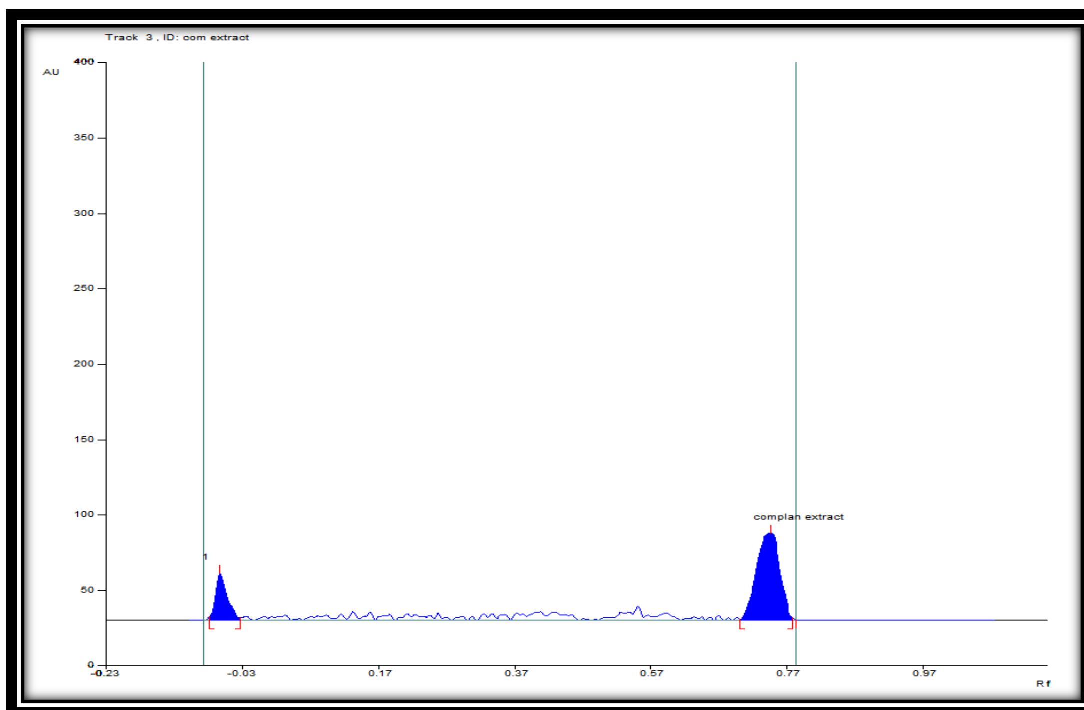


Fig 27: Chromatogram for dairymilk extract

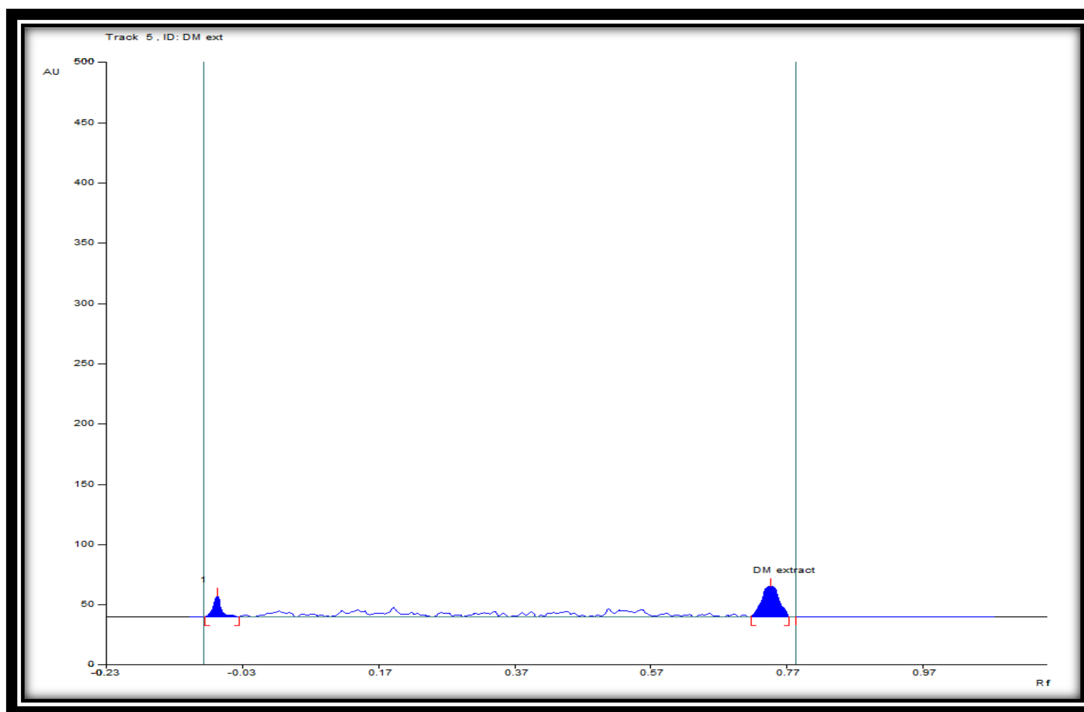


Fig 28: Chromatogram for kitkat extract

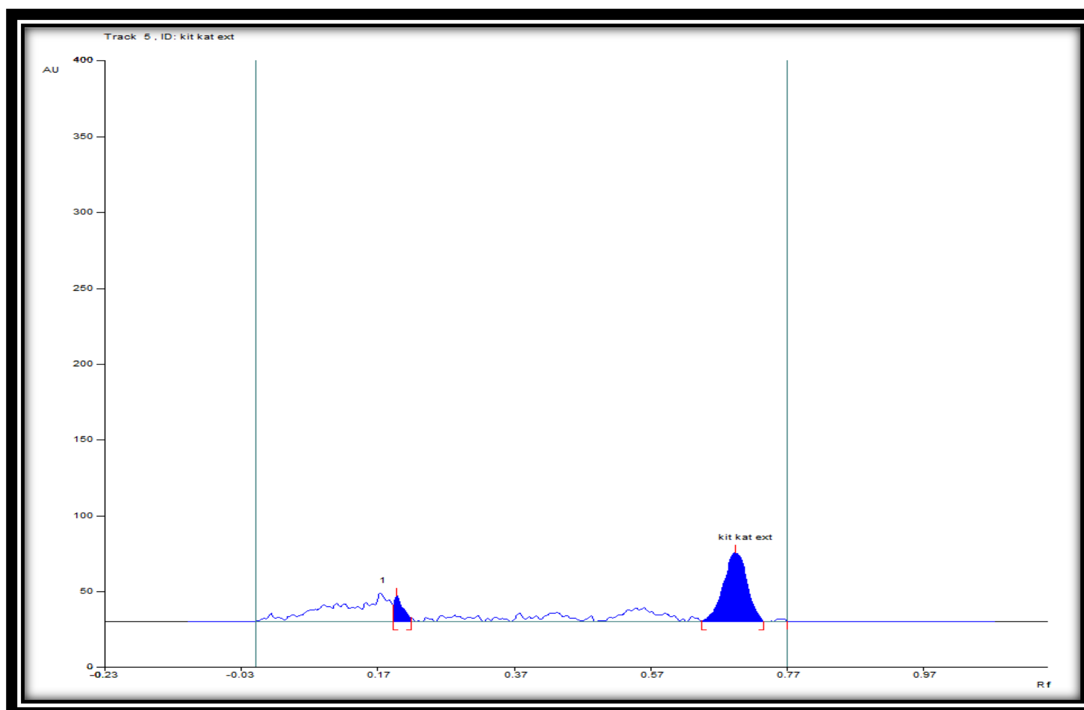


Fig 29: Chromatogram for munch extract

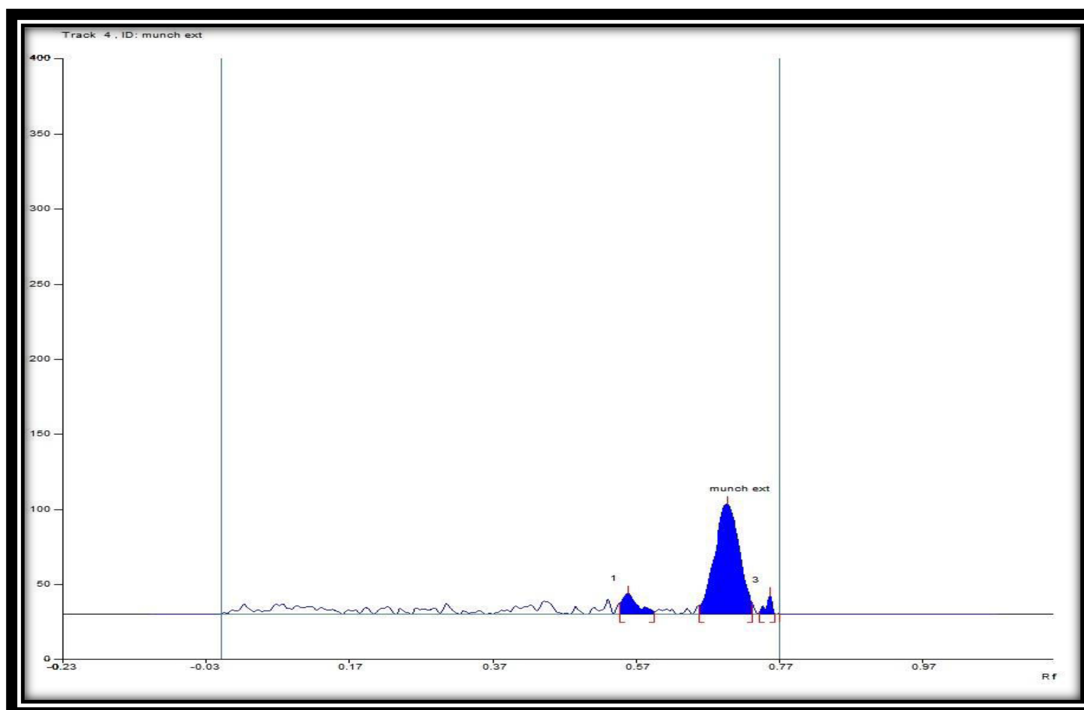


Table 20: % of BHA present in the extracted food stuffs

Food products	% of BHA	Acceptable limit of BHA (FDA guidelines)[26]
Chocos	0.0012	
Complan	0.00114	
Dairy milk	0.0000161	
Kit Kat	0.0000595	0.02%
Munch	0.00090	
Five star	-	
Milky bar	-	

From the table 20 the amount of BHA present in the selected food stuffs was found to be within the acceptable limit as per FDA guidelines and was not present in milky bar and five star chocolate.

DEVELOPMENT OF VALIDATED RP-HPLC METHOD FOR THE ESTIMATION OF BUTYLATED HYDROXYANISOLE IN SELECTED FOOD STUFFS

Proper selection of chromatographic method depends upon the nature of the sample. Since BHA is polar in nature, reverse phase chromatography has been used because of its simplicity and solubility.

Different solvent such as methanol, acetone, ethanol and acetonitrile were tried. Butylated hydroxyanisole is readily soluble in acetonitrile and methanol. But its stability is more in acetonitrile. Hence acetonitrile was selected as a solvent of choice and used for further analysis.

While developing RP-HPLC method different mobile phase were tried and the results are shown in table 21. In order to obtain a better peak purity acetonitrile: water was selected as mobile phase.

Table 21: Selection of mobile phase

Mobile phase	Retention time(min)	Observation
Acetonitrile	2.8	Impure peak
Methanol	3.2	Impure peak
Methanol: water	6.2	Asymmetrical peak
Acetonitrile: water	4.02	Symmetrical peak

After selection of mobile phase the effect of ratio was studied using 50: 50, 70: 30, 20: 80, and 80: 20 %v/v under the fixed chromatographic condition. The results are shown in table 22. Finally acetonitrile: water in the ratio 80: 20 %v/v was selected to get a symmetrical peak with a purity index 1 at the retention time 4.02 min.

Table 22: Effect of mobile phase ratio

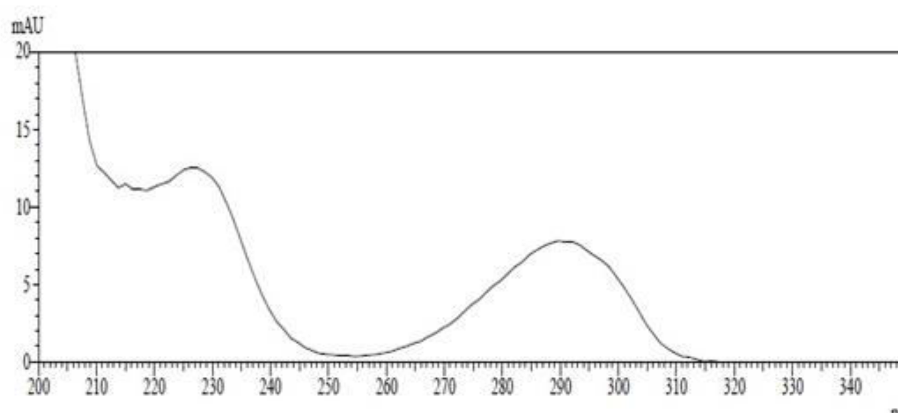
Ratio (%v/v)	Observation
50: 50	Broadening
70: 30	Asymmetric peak
20: 80	Broadening
80: 20	Symmetrical peak

Keeping the mobile phase ratio as 80:20 %v/v, the chromatograms were recorded at different flow rates like 0.5ml/min, 1.0ml/min and 1.5 ml/min. With flow rates of 0.5ml/min and 1.5ml/min, peak was not symmetrical but a flow rate at 1.0ml/min gave good symmetrical peak and hence used for further study. The results are shown in table 23. The spectrum of BHA is shown in figure 30.

Table 23: Effect of flow rate

Flow rate (ml/min)	Observation
0.5	Tailing
1.0	Symmetric peak
1.5	Impure peak

Figure 30: UV spectrum of Butylated hydroxyanisole using HPLC system



After the development of RP-HPLC method for the estimation of BHA, validation of the method was carried out. For all the methods % RSD was calculated.

From the stock solution of BHA (1000µg/ml) standard solutions were prepared in the concentration range 0.1–1.0µg/ml using acetonitrile. The solutions were injected into HPLC column and chromatograms were recorded. The standard chromatograms are shown in figure 31- 40. The peak area results are given in table 24 and the corresponding calibration graph was constructed between peak area versus concentration and shown in figure 41.

Figure 31: Chromatogram of butylated hydroxyanisole (0.1 µg/ml)

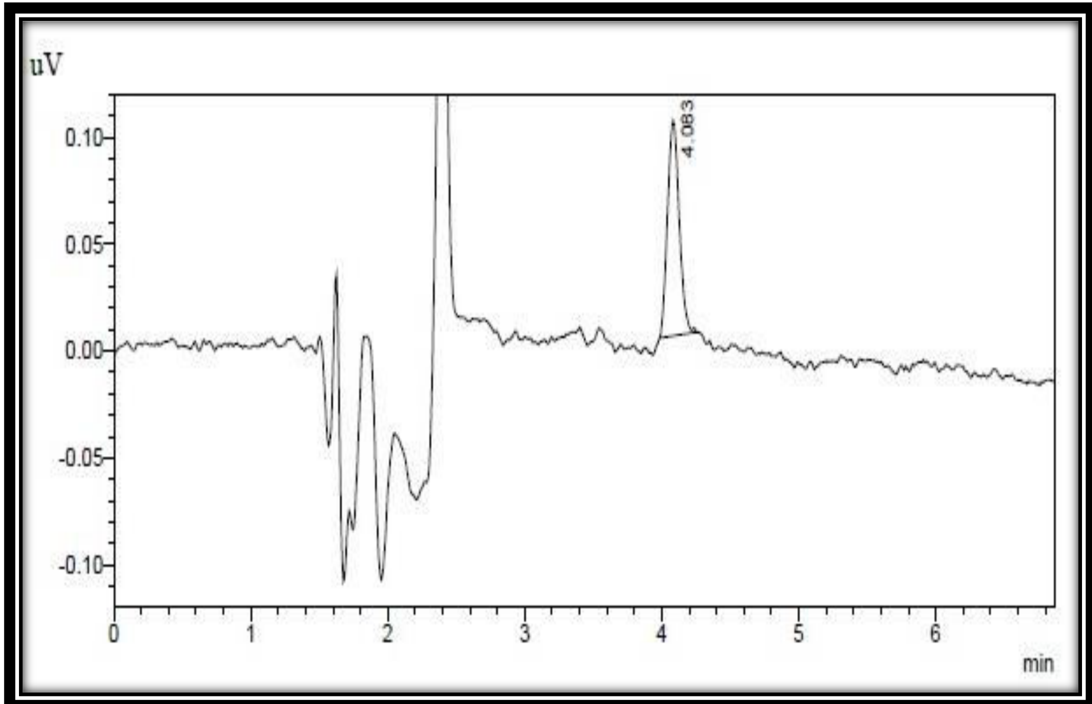


Figure 32: Chromatogram of butylated hydroxyanisole (0.2µg/ml)

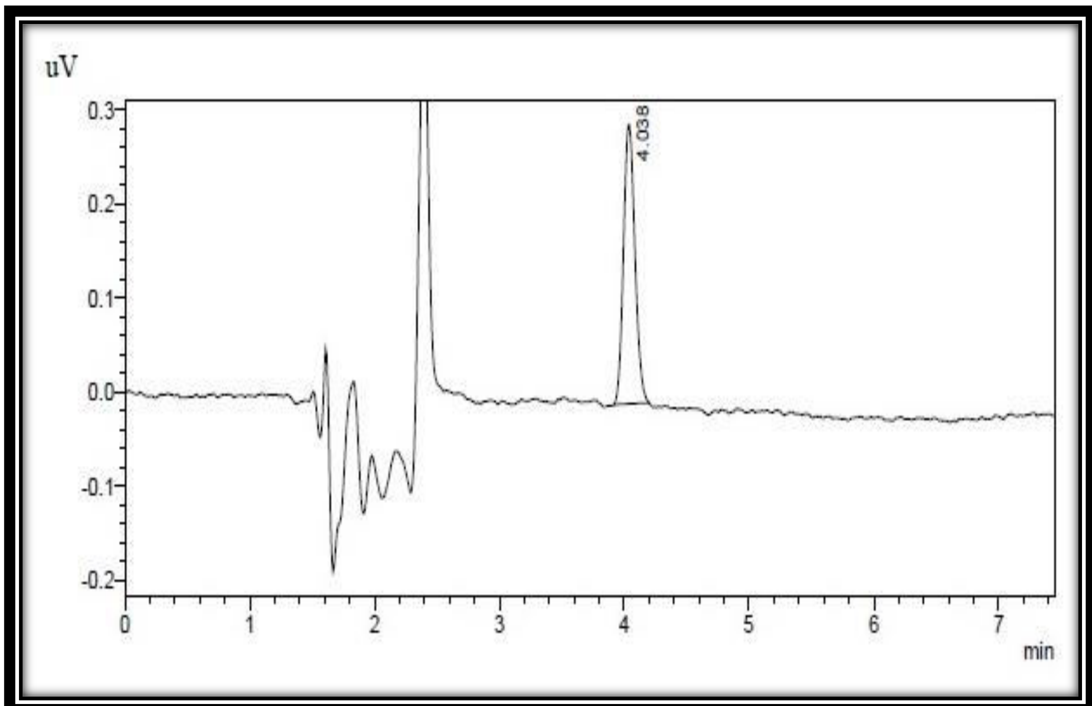


Figure 33: Chromatogram of butylated hydroxyanisole (0.3µg/ml)

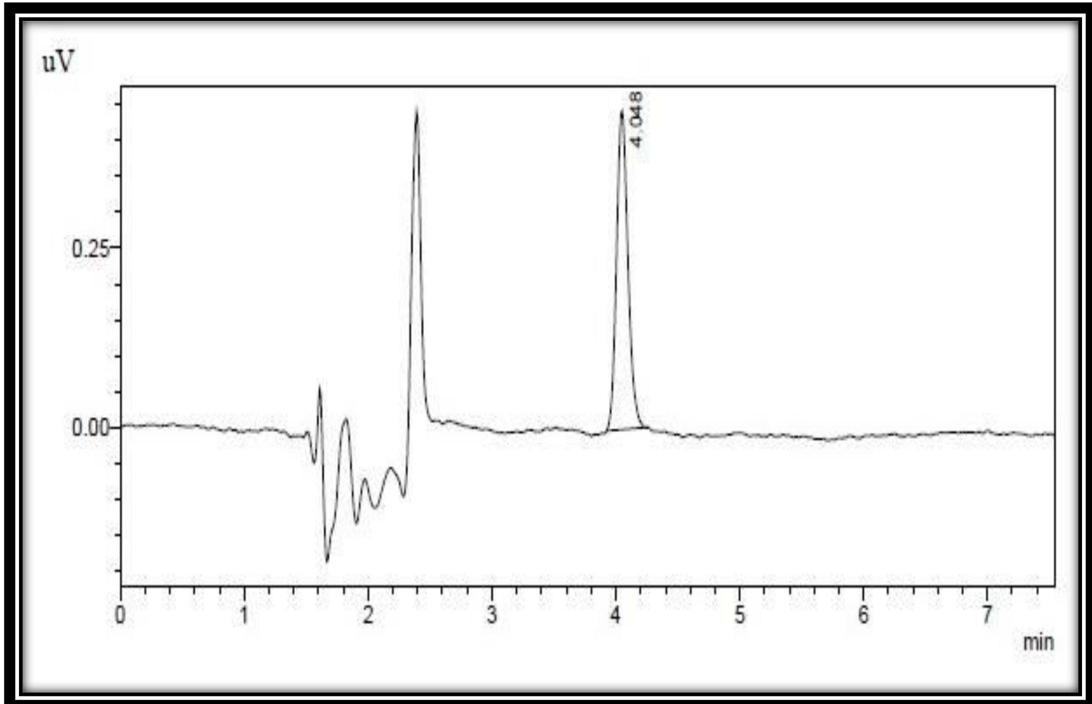


Figure 34: Chromatogram of butylated hydroxyanisole (0.4µg/ml)

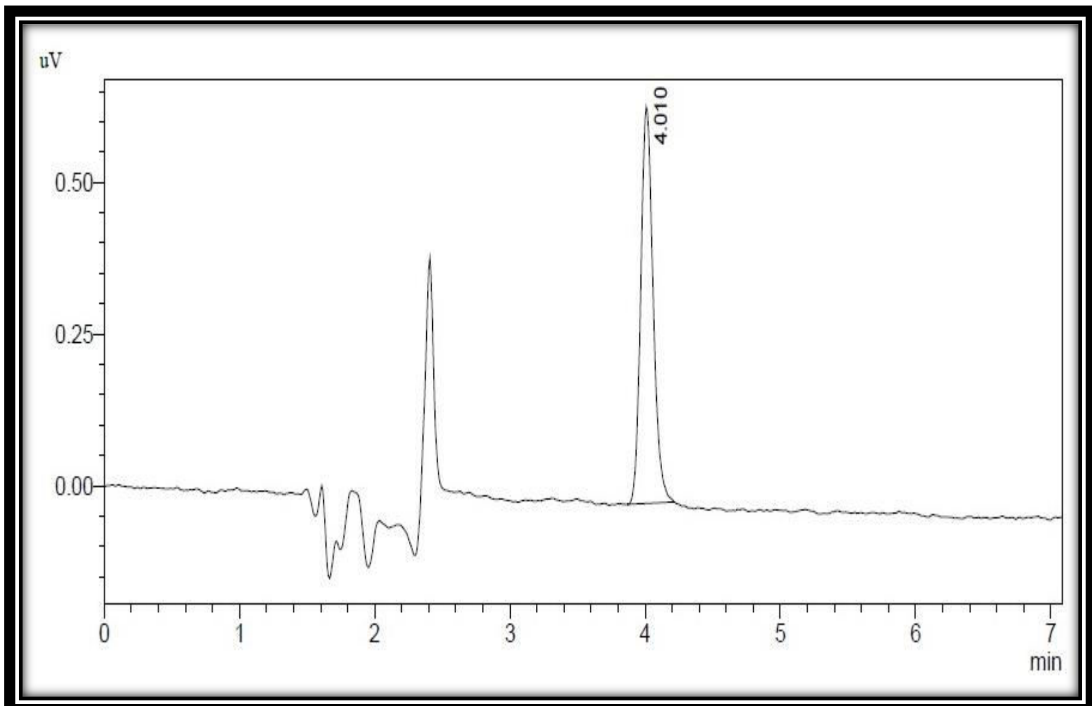


Figure 35: Chromatogram of butylated hydroxyanisole (0.5µg/ml)

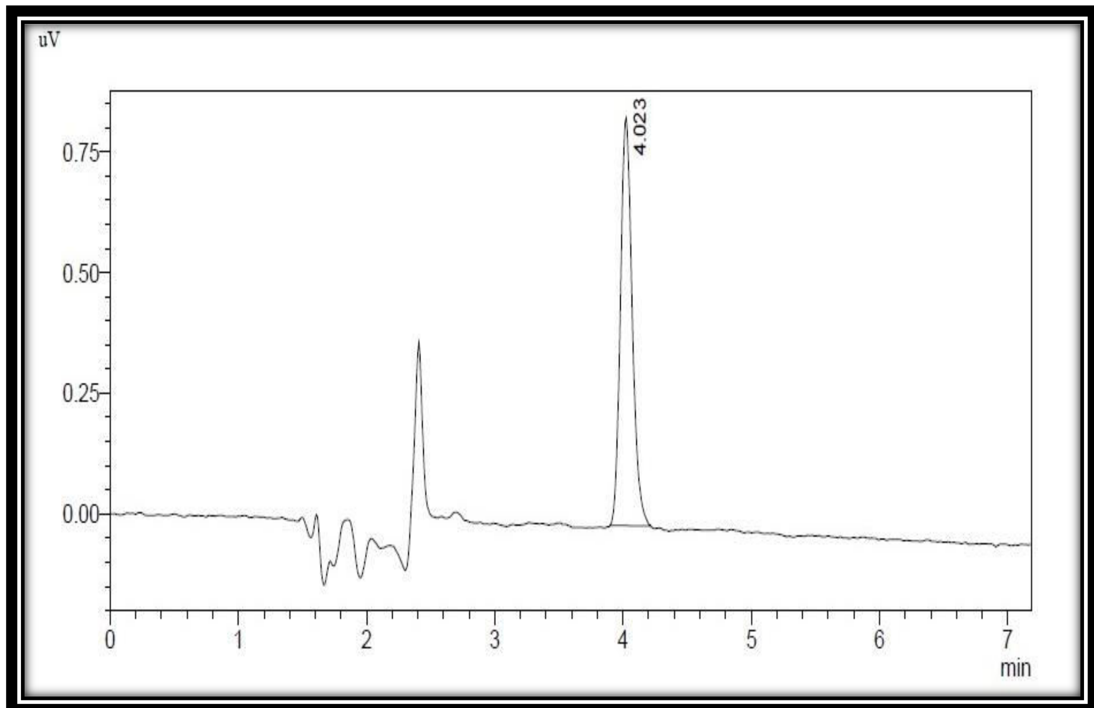


Figure 36: Chromatogram of butylated hydroxyanisole (0.6µg/ml)

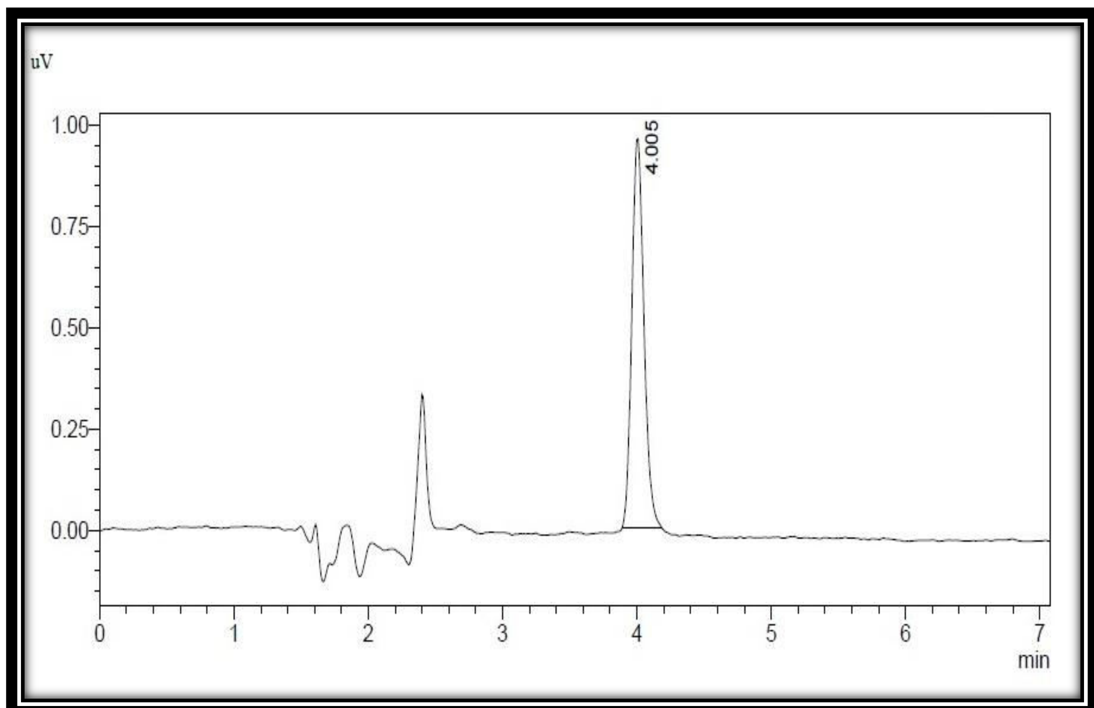


Figure 37: Chromatogram of butylated hydroxyanisole (0.7µg/ml)

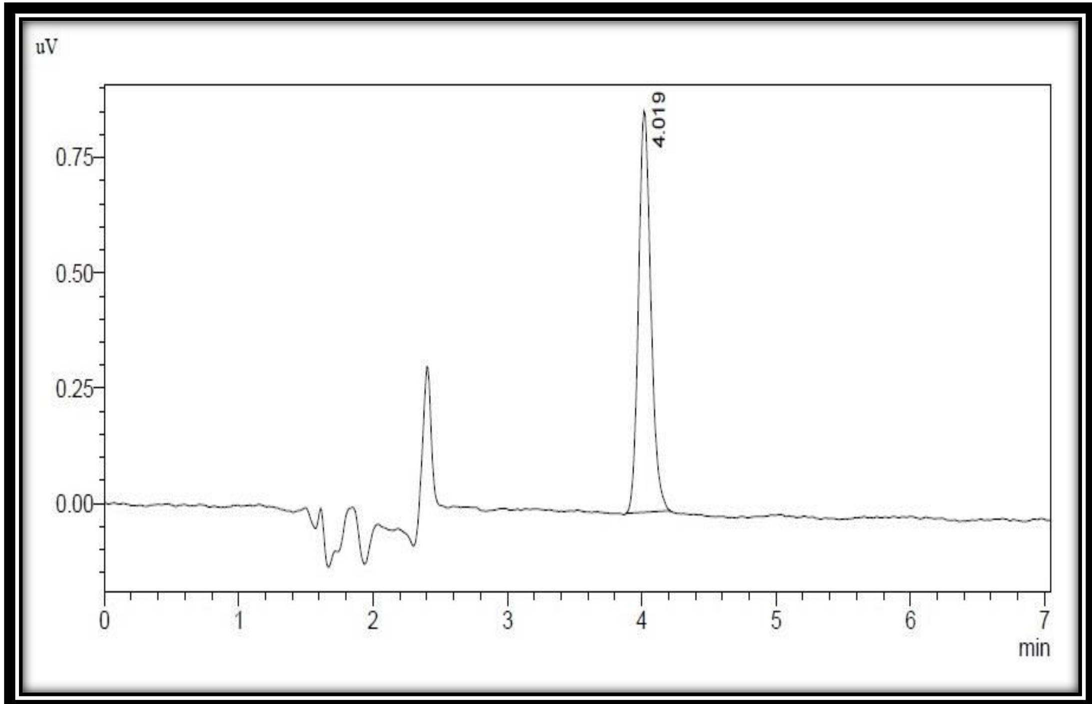


Figure 38: Chromatogram of butylated hydroxyanisole (0.8µg/ml)

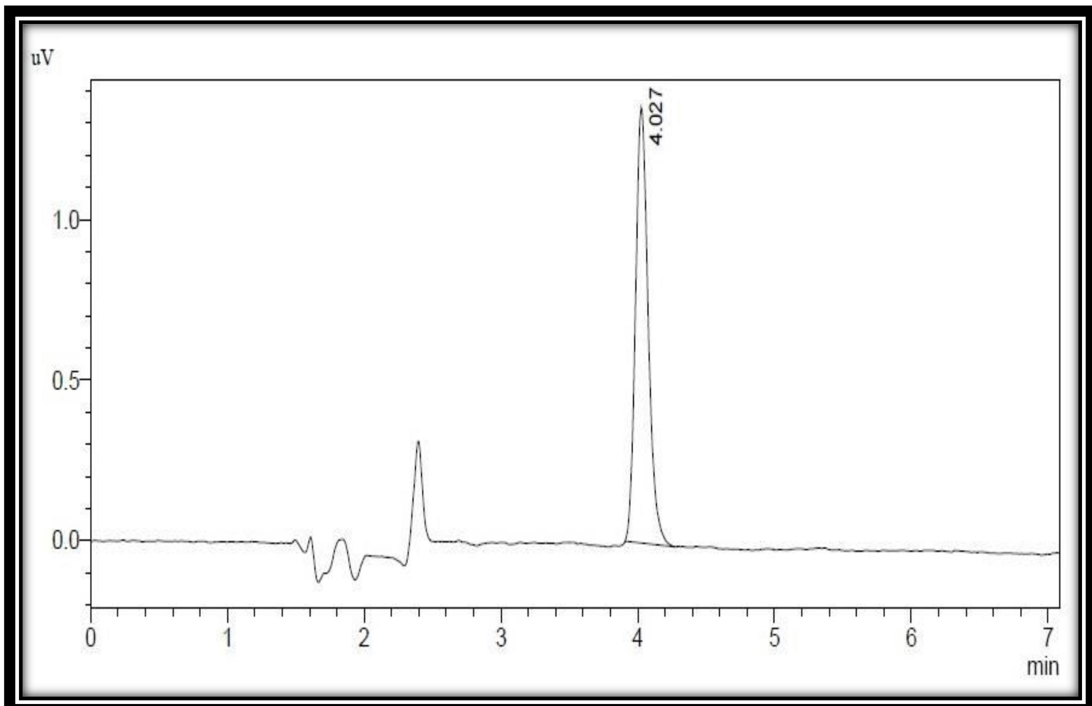


Figure 39: Chromatogram of butylated hydroxyanisole (0.9µg/ml)

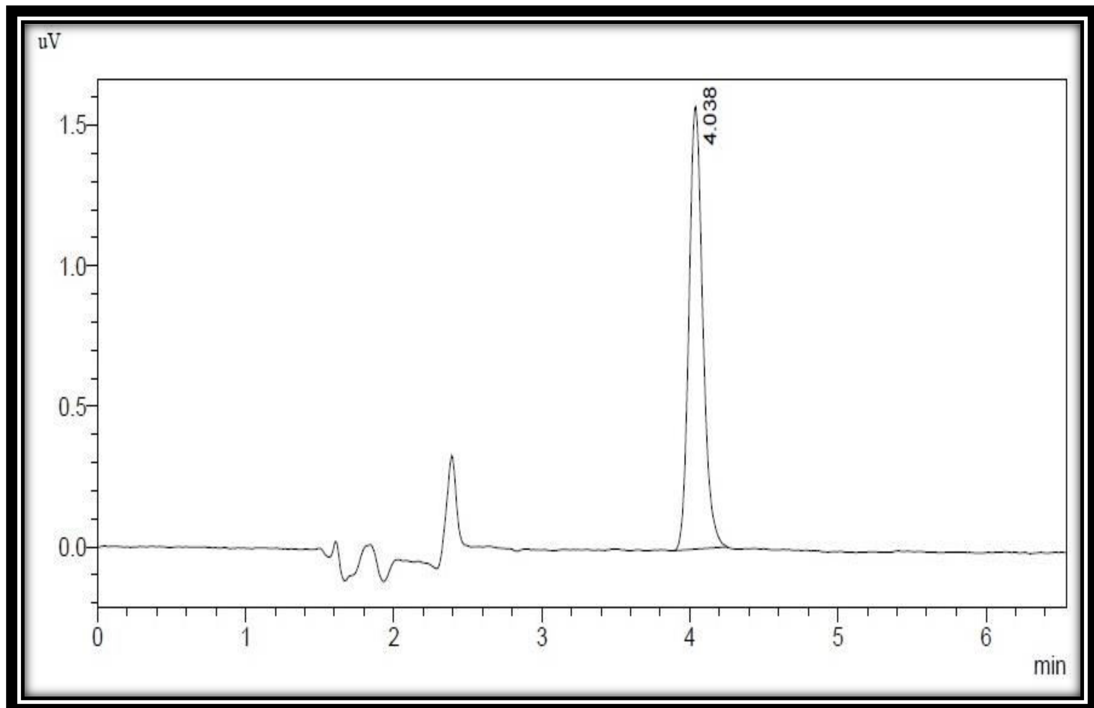


Figure 40: Chromatogram of butylated hydroxyanisole (1.0µg/ml)

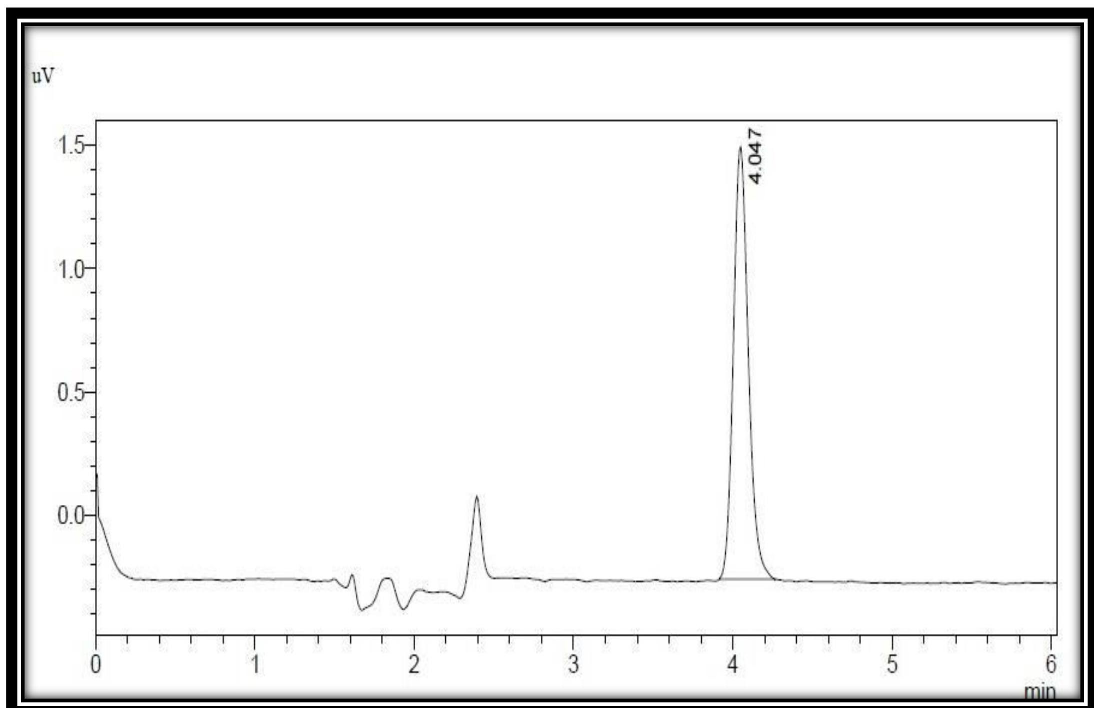
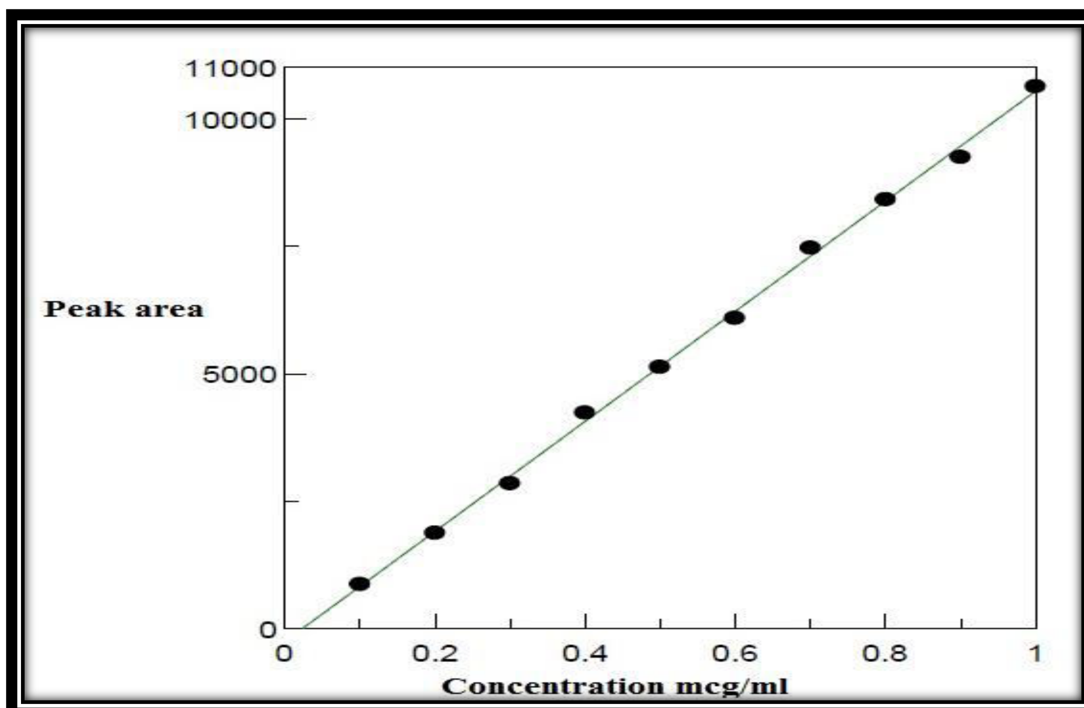


Table 24: Calibration data of Butylated Hydroxyanisole

Concentration (µg/ml)	Peak area
0.1	872
0.2	1874
0.3	2845
0.4	4231
0.5	5134
0.6	6090
0.7	7466
0.8	8421
0.9	9248
1.0	10638

Figure 41: Calibration graph of Butylated hydroxyanisole



Precision of the method was determined by repeatability of injection, intra day and inter day precision studies.

For this study a sample solution of concentration 0.5µg/ml of BHA was injected 6 times and its percentage RSD were calculated. The results are given in table 25.

Table 25: Repeatability of injection

Concentration (µg/ml)	Peak area	%RSD*
0.5	5158	0.45
	5171	
	5163	
	5187	
	5123	
	5134	

***Mean of six determinations**

Six multiple solution of same concentration (0.7µg/ml) of BHA was prepared, injected into the HPLC column and the response for each injection was measured. The percentage RSD was calculated and the results are shown in table 26.

Table 26: Intra day precision

Concentration (µg/ml)	Peak area	%RSD*
0.7	7469	0.07
	7471	
	7463	
	7473	
	7480	
	7475	

***Mean of six determinations**

Inter-day precision was carried out by injecting a concentration (0.7µg/ml) of the sample solution for two days and the percentage RSD was calculated and the results are given in table 27.

Table 27: Inter-day Precision

Concentration (µg/ml)	Days	Peak area	%RSD*
0.7	I	7469	0.07
		7471	
		7463	
		7473	
		7480	
		7475	
	II	7516	0.26
		7572	
		7605	
		7532	
		7528	
		7580	

***Mean of six determinations**

Accuracy of the method was carried out to know about the reliability and accuracy of the method. The concentration of BHA present in the 50% and 100% level were determined. The procedure was repeated 6 times and % recovery was calculated using the formula and the results are shown in table 28.

$$\% \, recovery = \frac{(amount\ of\ drug\ found\ after\ the\ addition\ of\ the\ standard\ drug) - (amount\ of\ drug\ found\ before\ the\ addtion\ of\ std.drug)}{amount\ of\ standard\ drug\ added} \text{ x } 100$$

Table 28: Recovery studies

Substance	% Recovery		%RSD*	
	50	**100**	**50**	**100**
Butylated hydroxyanisole	101.8	100.1	0.75	0.94

***Mean of six determinations**

The LOD and LOQ were calculated using the equation,

$$LOD = 3.3 \times \sigma / S$$

$$LOQ = 10 \times \sigma / S$$

Where, σ is the standard deviation of y intercepts of regression line, & S is slope of calibration curve. LOD was found to be 0.02 µg/ml and LOQ was found to be 0.08 µg/ml.

The study was confirmed to be robust as the retention time and peak area was not much affected by introducing slight changes in the experimental conditions, that is shown in table 29 and 30.

Table 29: Effect of flow rate

Flow rate (ml/min)	R_T (min)
0.9	3.98
1.0	4.02
1.1	4.10

Table 30: Effect of mobile phase ratio

Mobile phase ratio(ACN: water)	R_T (min)
79:21	3.96
80: 20	4.02
81:19	4.12

The system suitability parameters such as tailing factor, and peak asymmetry were calculated from the standard chromatograms and was found to be 1.174 and 1.14.

The standard solution of BHA (0.5µg/ml) was kept under room temperature. It was injected periodically. Stability was studied by looking for any change in retention time, peak shape and reduction in peak area when compared to chromatogram of freshly prepared standard solution. It was observed that BHA was found to be stable for about 7 days when kept under room temperature.

Sample preparation is an important procedure in chromatographic application. One of its main aim is to remove interfering matrix components and particulates as well as to concentrate analytes of interest to enhance the sensitivity. N-hexane was selected because it is having capability to remove fats and oil containing products from various food products than petroleum ether. The extracting efficiency was found to be good in acetonitrile: n-hexane compared to methanol: n-hexane. Hence acetonitrile: n-hexane was selected as the extracting solvent for BHA from other ingredients like fats, oils etc. To the extracted solution 0.5µg/ml of standard solution was added and the chromatograms were recorded after injecting the sample, corresponding chromatograms are shown in figure 42 – 48. From the peak area obtained % BHA was calculated for each food stuffs and the results are given in table 31.

Figure 42: Chromatogram of chocos extract

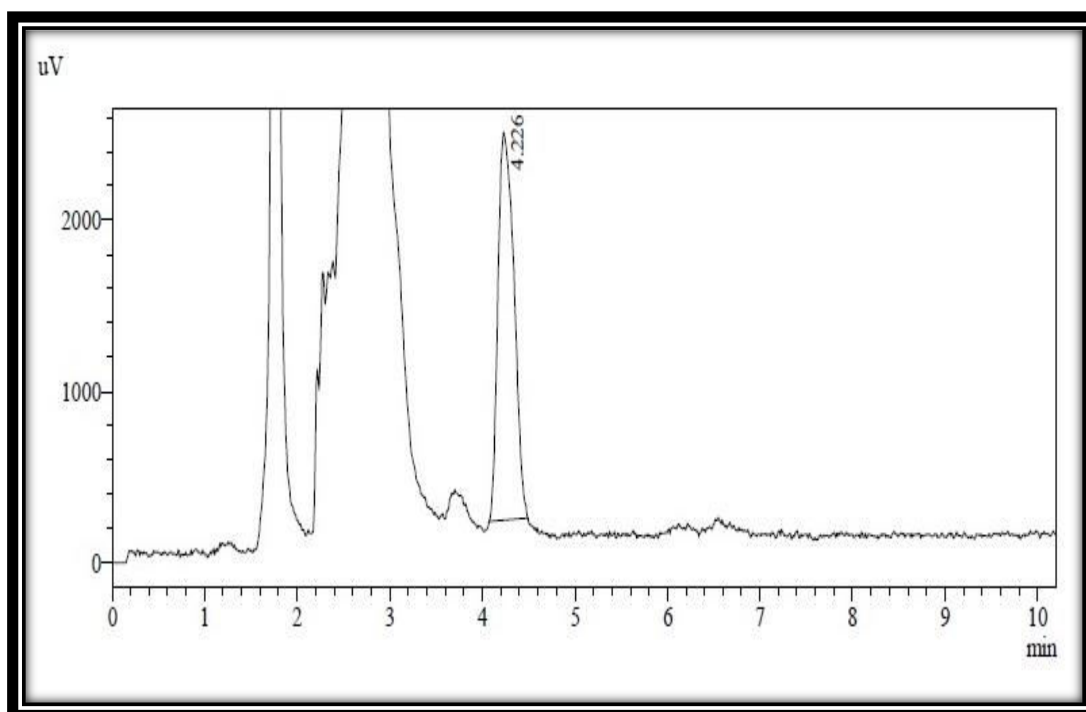


Figure 43: Chromatogram of complan extract

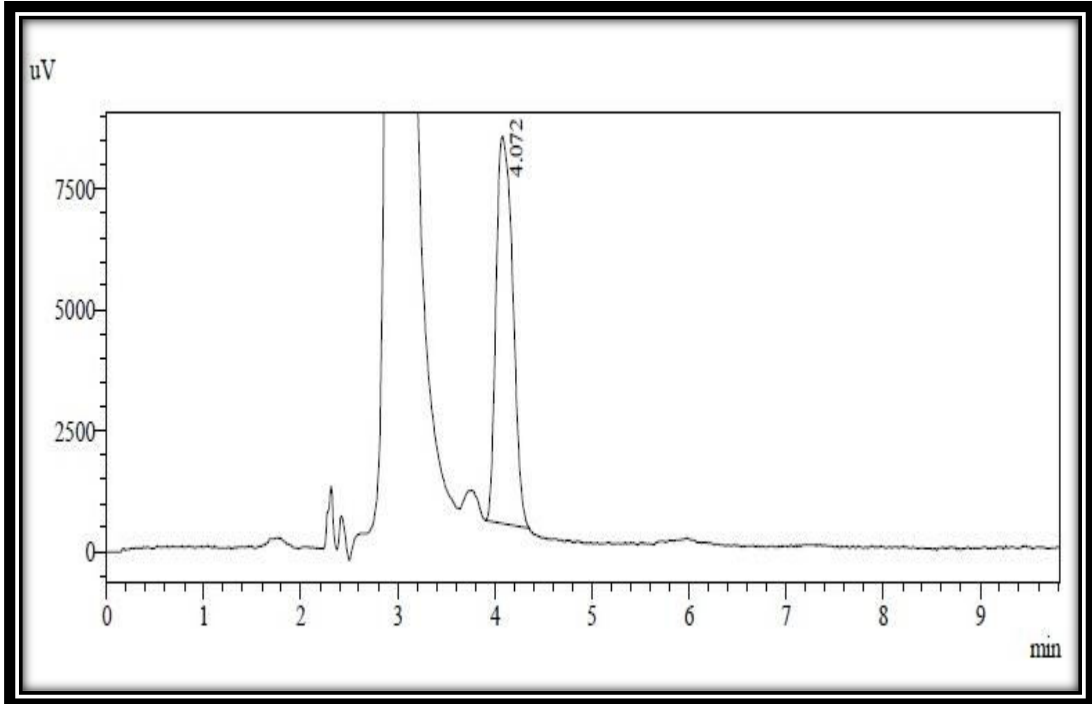


Figure 44: Chromatogram of kit kat extract

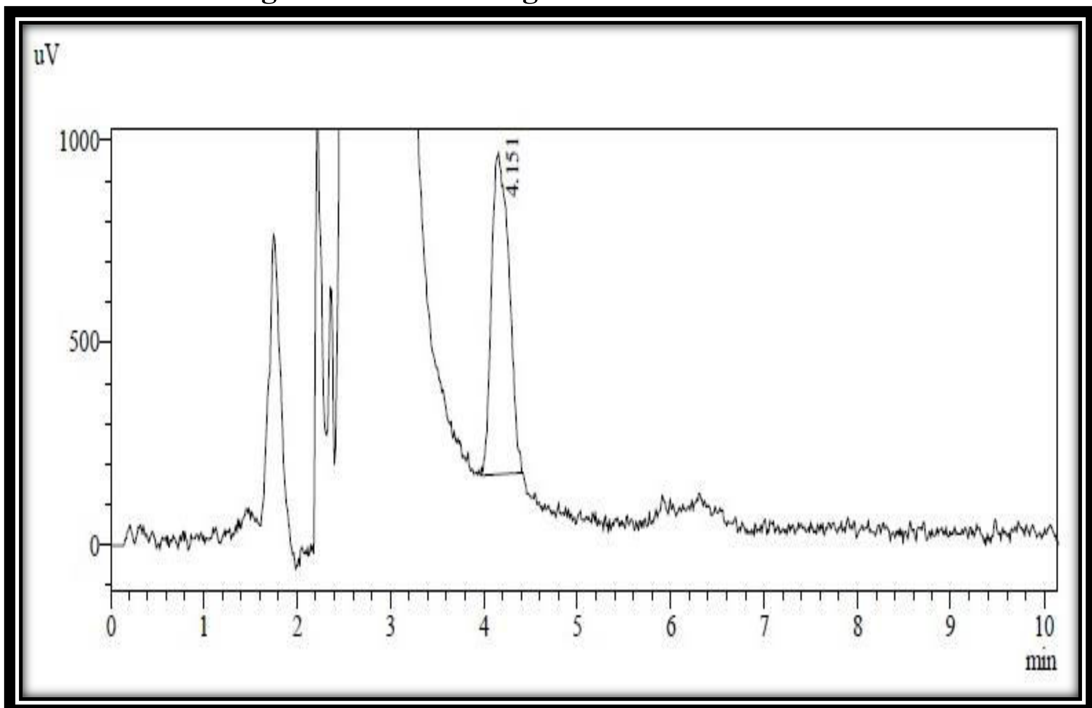


Figure 45: Chromatogram of milky bar extract

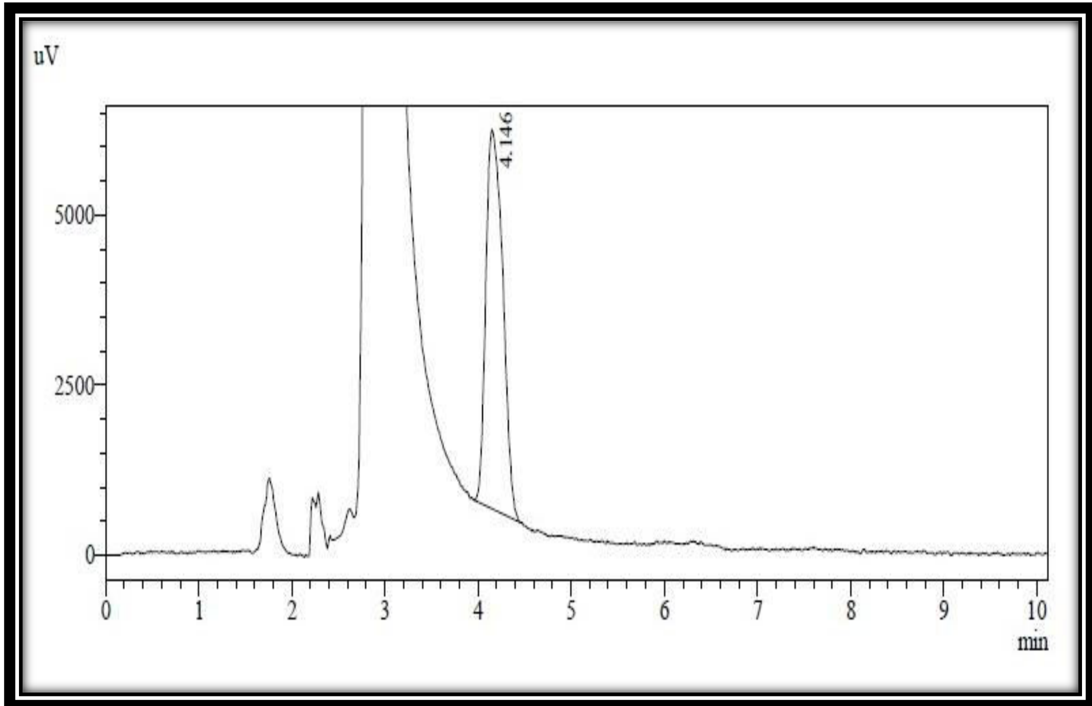


Figure 46: Chromatogram of munch extract

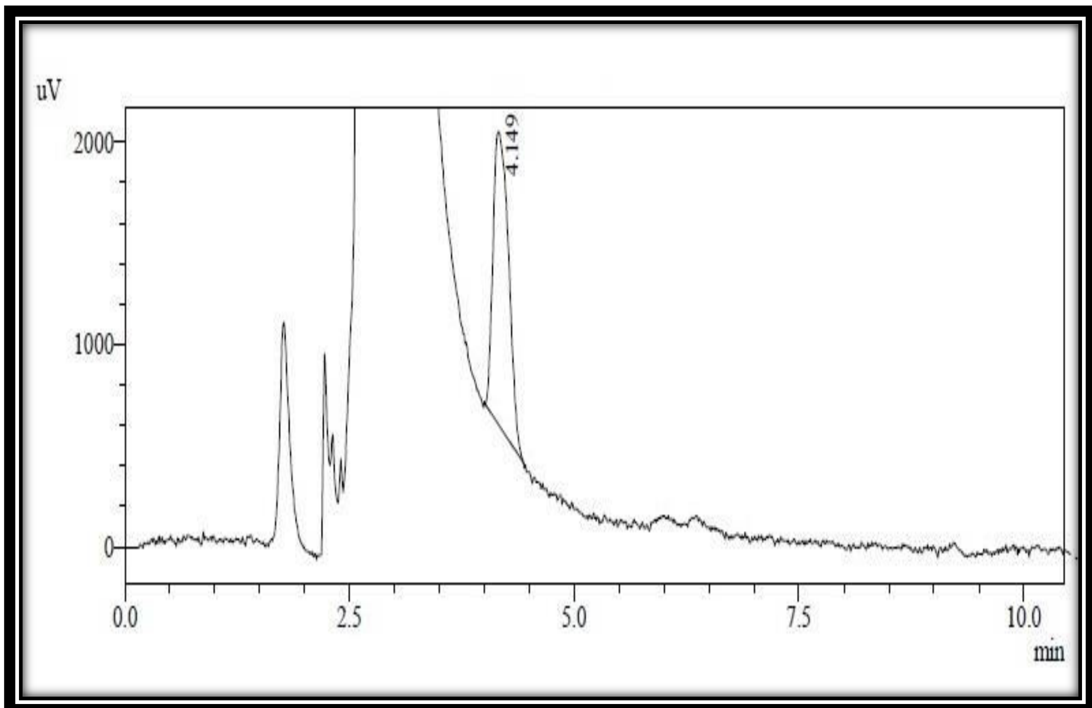


Figure 47: Chromatogram of five star extract

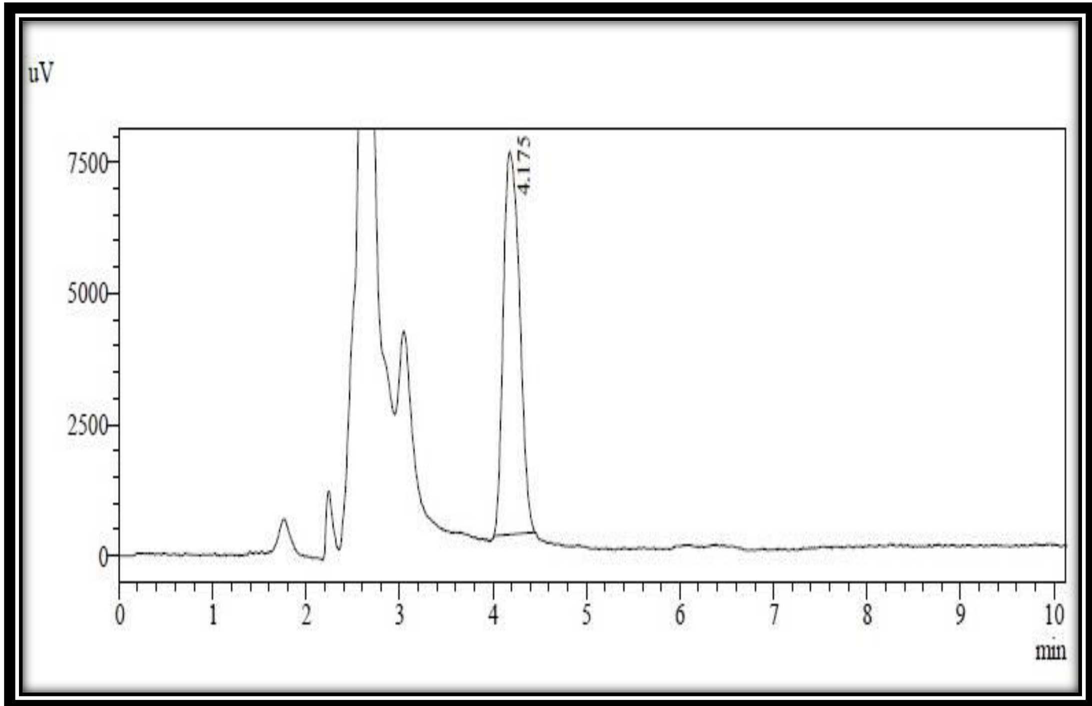


Figure 48: Chromatogram of dairy milk extract

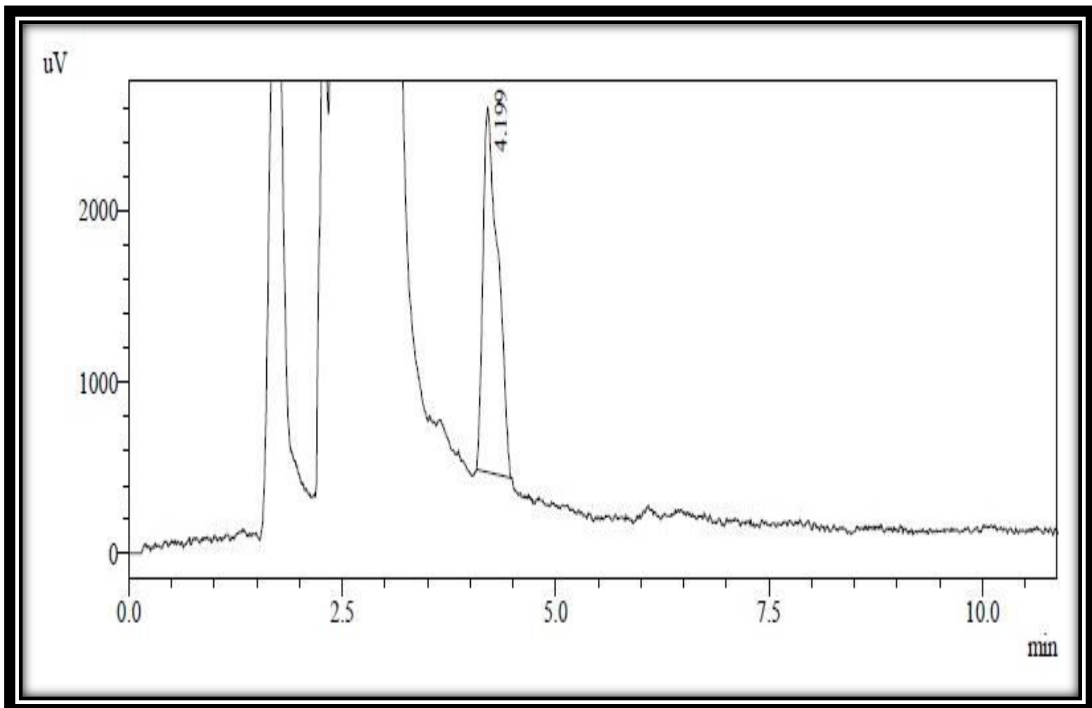


Table 31: % of BHA present in the extracted food stuffs

Food products	% of BHA	Acceptable limit of BHA (FDA guidelines) [26]
Chocos	0.0134	0.02%
Complan	0.01426	
Kit Kat	0.000926	
Milky bar	0.000830	
Five star	0.000535	
Dairy milk	0.0000476	
Munch	0.000497	

From the table 31 it was found that the % BHA content in the selected food stuffs was found to be within the acceptable limit as per FDA guidelines.

SUMMARY AND CONCLUSION

An attempt has been made to develop validated UV spectroscopic and stability indicating HPTLC methods for the estimation of isradipine in bulk drug and development of validated RP-HPLC and RP-HPTLC methods for the estimation of butylated hydroxyanisole in selected food stuffs.

A simple and sensitive UV spectroscopic method has been developed for the estimation of isradipine in bulk drug. Isradipine exhibited maximum absorbance at 326nm in methanol. Beer's law was obeyed in the concentration range of 5- 50µg/ml. The % recovery values close to 100% indicated the accuracy of the method developed. More over the data of analysis was supported by RSD values.

HPTLC method was developed for isradipine in bulk drug on silica gel 60 F_{254} TLC plates using mobile phase of toluene: methanol: glacial acetic acid (9:1:0.05% v/v/v) and detection was carried out at 331nm. The R_f value of isradipine was 0.34 (±0.03). The percentage recovery of isradipine was found to be 101.3± 0.35 for 50% and 101.0± 0.36 for 100% with coefficient variation 0.9994. The drug was subjected to acid hydrolysis, alkaline hydrolysis, oxidation, thermal and photolytic degradation. The degradation study indicated isradipine was susceptible to acid hydrolysis and alkaline hydrolysis. The degradation products were well resolved from the pure drug with significant differences in R_f values. The R_f value of isradipine was 0.34± 0.03 and acid degradants were appeared at the R_f value of 0.15 and 0.51. The R_f value of alkaline degradant was 0.20. The comparative study is tabulated in table 32.

Table 32: Comparison of UV and HPTLC method for isradipine

Parameters	UV	HPTLC	Reported [18]
Linearity (µg/ml) or (µg/band)	5- 50	1-6	50- 400
r	0.9997	0.9994	0.999
LOD (µg/band) or (µg/ml)	-	0.018	0.30
LOQ (µg/band) or (µg/ml)	-	0.056	0.10
Molar absorptivity L mol^{-1} cm^{-1}	9.3 x 10^3	-	-
% recovery ±% RSD*			
50	102.0± 0.12	101.3± 0.35	99.9±0.12
100	101.5± 0.51	101.0± 0.36	101.0±0.008
Precision (% RSD)*			
Repeatability	0.58	0.30	0.012
Intraday	0.51	1.20	0.006
Interday	0.8	1.4	0.004

*Mean of six determinations

Both the methods developed for determination of isradipine was found to be more sensitive than the reported method. [18]

A convenient and rapid RP-HPLC method has been developed for the estimation of butylated hydroxyanisole in several food products. Separation was achieved by using Hibar 250- 4, 6 lichrospher 5µm column, using a mobile phase consisting of acetonitrile: water (80: 20%v/v) at a flow rate 1.0ml/min. The detection wavelength was selected at 290nm by using PDA detector. The calibration curves are linear in the range of 0.1 to 1µg/ml of butylated hydroxyanisole. The mean recovery obtained by using these methods was found to be 101.8± 0.75% for 50% and 100.1± 0.94% for 100% with coefficient of variation 0.999 for RP-HPLC method. The developed method could be used as fingerprint for the measure of BHA present in selected food products under optimum parameters. The method is fast, accurate, sensitive, provide excellent recoveries, convenient and effective for the finger printing and quantification of BHA for routine analysis in various food stuffs.

RP-HPTLC method was developed for butylated hydroxyanisole on pre-coated silica gel RP-18 F_{254} aluminium plate using mobile phase comprising of glacial acetic acid: acetonitrile: water (5: 4 : 1) and the detection was carried out at 290nm. The R_f value of butylated hydroxyanisole was 0.70± 0.03. The percentage recovery of BHA was found to be 100.28± 0.23 and 101.17± 1.30 for 50% and 100% with coefficient of variation 0.9992. The developed method can be used to fingerprint the BHA present in selected food products under optimum parameters. The proposed RP- HPTLC method has high degree of repeatability and will provide fast and cost-effective quantitative control for routine analysis of BHA in selected food products.

Comparison of validated parameters for the developed methods is summarized in table 33.

Table 33: Comparison of RP-HPLC and RP- HPTLC method for BHA

Parameters	RP-HPLC	RP-HPTLC
Linearity (µg/ml) or (µg/band)	0.1-1	1- 3.5
r	0.9990	0.9992
LOD (µg/ml) or (µg/band)	0.02	0.03
LOQ (µg/ml) or (µg/band)	0.08	0.10
% recovery ± % RSD* 50 100	 101.18± 0.75 100.1± 0.94	 100.28± 0.23 101.17± 1.30
Precision (% RSD)*		
Repeatability	0.45	0.72
Intraday	0.07	0.55
Interday	0.26	0.32

*Mean of six determinations

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