Original Research Article

STABILITY INDICATING HPLC METHOD FOR ESTIMATION OF THYMOQUINONE IN NASAL SIMULATED FLUID: METHOD DEVELOPMENT AND VALIDATION

ABSTRACT

Introduction: A simple rapid and precise HPLC method was developed for estimation of TH in nasal simulated fluid and stability was assessed in various stressed conditions.

Method: Chromatographic separation of TH in nasal simulated fluid was done using HPLC AS-4050 coupled with Jasco UV 2075 Plus detector, Jasco LC-Net 11/ADC valve, Jasco PU-2080 pump and hypersil gold C18 (250x6x5 µm) column, ChromNAV 2.0 Chromatography Data System software with mobile phase as acetonitrile: water (65:35) and acetonitrile: NSF (60:40) at a flow rate of 1ml/min and having run time of 10 min with loop volume of 20 µl and detection wavelength of 252nm. The method was validated for accuracy, precision, linearity, specificity, and sensitivity in accordance with ICH (Q2B) guidelines.

Results: The results of all the validation parameters were found to be within the acceptable limits. The calibration plots were linear over the concentration ranges from 2 to 14µg/ml. The accuracy and precision were found to be between 97.04±0.112 to101.081±0.0191and ≤2% for three drugs. Developed method was successfully applied for the determination TH in nasal simulated fluid and recovery was found to be >98% for three drugs. The degradation products produced as a result of stress studies did not interfere with drug peak.

Conclusion: The developed method was found to be simple, specific, economic, reliable, accurate, precise, and reproducible used as a quality control tool for analysis of pure thymoquinone in nasal simulated fluid.

Keywords: Thymoquinone, Nasal simulated fluid (NSF), HPLC, Stress degradation, Method validation.

1.0 INTRODUCTION

Past two decades, witnessed an enormous research to divulge the pharmacological actions of Thymoquinone (TH) which profusely found in the seeds of Nigella sativa L (Ranunculaceae family)

and chemically is 2-methyl-5-isopropyl-1, 4-benzoquinone and monoterpene in nature[1-3]. The literature reveals that TH possess various pharmacological properties includes, anti-convulsant, anti-microbial, anti-cancer, anti-histaminic, anti-diabetic, anti-inflammatory, and anti-oxidant activity and is capable of influencing and altering various molecular and signalling pathways especially in inflammatory and degenerative diseases together with cancer[4–7]. Glioblastoma multiforme (GBM) is a most aggressive form of brain tumour, and is challenging to treat because of its devastating nature and site of tumours. Various studies have shown that TH induces changes in several tumorigenic processes and counteract carcinogenesis, malignant growth, invasion, migration, and angiogenesis and mainly is multitargeting in nature[6,8]. The major challenge in treating GBM, cerebral ischemia is blood brain barrier (BBB) and potential of TH to enter brain via nasal pathway due to volatile nature could be advantage in overcoming blood-brain barrier. [1,10]. To the best of our knowledge at present no reliable method has been reported to estimate the TH in the nasal fluid and literature also suggests that, there is no HPLC (High Pressure Liquid Chromatography) method reported for the estimation of TH in nasal simulated fluid. Hence the present work focuses on developing and validating a standard HPLC method for TH in nasal simulated fluid and assessing the stability in various conditions.

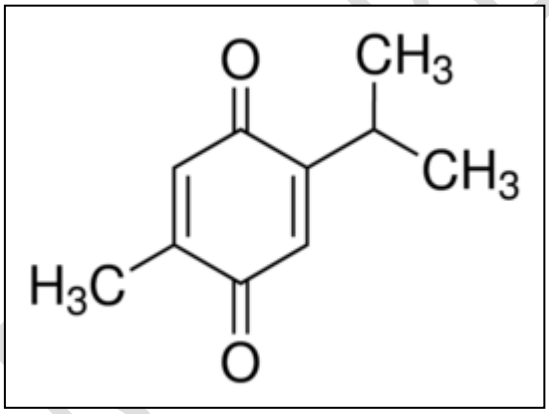

Figure 1. Structure of Thymoquinone.

2.0 MATERIALS AND METHOD

2.1 Materials

Thymoquinone was purchased from Cayman Chemicals Company, USA, Purified water was obtained from a Millipore Direct-Q 3 U.V. Acetonitrile of HPLC grade, and all other chemicals were of analytical grade.

2.2 Method

2.2.1 Instrumentation

The instruments employed in the study were Jasco autosampler HPLC AS-4050 coupled with Jasco UV 2075 Plus detector, Jasco LC-Net 11/ADC valve, Jasco PU-2080 pump and

hypersil gold C18 (250x6x5 µm) column,ChromNAV 2.0 Chromatography Data System software, sonicator-Bio Technics Mumbai, India, analytical balance; pH meter-Systronics, Ahmadabad, India.

2.2.2 Stock and Working Solution Preparation

TH was accurately weighed 10 mg and was transferred to a 10ml volumetric flask containing solvent (acetonitrile: water 65:35) and was dissolved into it and volume was made up with the same solvent at same ratio of 65:35 v/v acetonitrile and water. The working standard solution of different concentrations were prepared by suitable dilution of the stock solution with the mobile phase.

2.2.3 Preparation of Nasal simulated fluid

Accurately weighed NaCl (7.45 mg/ml), KCl (1.29 mg/ml) and Ca-Cl22H2O (0.32 mg/ml) were dissolved in 1000 ml of distilled water to produce nasal simulated fluid (NSF), pH of solution was adjusted to 6.75 using triethanol amine[11].

2.2.4 Chromatographic Conditions

The chromatographic separation was done using Jasco autosampler HPLC AS-4050 coupled with Jasco UV 2075 Plus detector, Jasco LC-Net 11/ADC valve, Jasco PU-2080 pump and hypersil gold C18 (250x6x5 µm) column,ChromNAV 2.0 Chromatography Data System software with mobile phase as acetonitrile: water (65:35) and acetonitrile: NSF (60:40) at a flow rate of 1ml/min and having run time of 10 min with loop volume of 20 µl and detection wavelength of 252nm[12].

2.2.5 Optimization of the method

The target was to achieve complete separation and highest peak resolution of thymoquinone and optimization was done by varying several parameters like mobile phase solvents and its composition. The stability of NSF in terms of pH was critically monitored and all other chromatographic conditions were kept constant.

2.3 Method Validation

The validation of developed method was done as per the ICH guidelines with the help of parameters like linearity, sensitivity, accuracy, precision and stability of thymoquinone[13].

2.3.1 Linearity

To assess the linearity of developed method linear regression analysis and least square method was employed and the linearity of TH was found in concentration range of 2-14 µg/ml. The calibration was done by preparing the serial dilution (2, 4, 6, 8, 10, 12, 14 µg/ml)

from the stock solution using the mobile phase and after spiking the peak area of each concentration was measured and calibration curve was plotted between peak areas against concentration of thymoquinone.

2.3.2 Robustness

The robustness of the developed method was checked by inducing trivial changes in the reported critical parameters like composition of mobile phase, flow rate and nasal simulated fluid pH. After modification of these parameters as if no substantial changes were seen in developed method.

2.3.3 Precision and Accuracy

The precision and accuracy of the developed method was checked with by subjecting to repeatability (intra-day) and intermediate precision (inter-day) at three different concentrations for 3 different days in replicates of three and were expressed in terms of relative standard deviation (RSD).

3.0 FORCED DEGRADATION STUDY

The stress degradation study was carried out by taking 10 mg of TH in 10ml of volumetric flask and adding 2ml of 1M hydrochloric acid for acid degradation, similarly 10ml of volumetric flask containing 10mg of drug in mobile phase was placed kept in oven at 80o C for thermal degradation studies and 10ml of volumetric flask containing 10mg of drug in mobile phase was kept in UV light 254 nm region for photodegradation study. The stressed samples were further diluted with mobile phase samples were into the HPLC[14].

4.0 RESULT AND DISCUSSION

The work mainly focused on developing and validating HPLC method for estimation in nasal simulated fluid and simultaneously importance was given on the stability of TH in the stressed condition including acid degradation, thermal degradation and photodegradation. The optimization of chromatographic was done by using trial and error method by varying the ratio of solvents for mobile phase and adjusting the pH of NSF. The initial run for standard TH was done is mobile acetonitrile: water (65:35) and further several trials were carried out in NSF mobile phase and finally the better resolution of TH was found at the ratio of acetonitrile: NSF (60:40) with very tailing factor showing identical symmetry and in acceptable numbers. Initially, as reported by N. Habib et,al.[15]estimation of TH using HPLC was done with slight modification the peak of highest resolution was obtain by optimizing the ratio of acetonitrile and methanol. Hence concerning the ability of TH in overcoming the hurdles of brain delivery it becomes necessary for estimating the drug in nasal

microenvironment and which was mimicked with the help of nasal simulated fluid. Therefore, considering the solubility of TH, acetonitrile was selected as the organic solvent along with NSF in the ratio of acetonitrile: NSF (60:40) and after several trials the stated ratio gave highest resolution with minimum tailing factor at a flow rate of 1ml/min and having run time of 10 min with loop volume of 20 µl and detection wavelength of 252nm. The robustness of the developed was assessed by making minor changes in the dependent factors like composition of mobile phase, flow rate and nasal simulated fluid pH and no significant changes were observed in the chromatographic separations. As shown in figure 2 the HPLC chromatograms for TH in acetonitrile: water (65:35) and acetonitrile: NSF (60:40) are shown, having sharply resoluted peak. System suitability was also checked by injecting TH using acetonitrile: NSF (60:40) as mobile phase and the retention time, tailing factor and theoretical plates for TH was observed along with percentage relative standard deviation (%RSD) of three consecutive injections for each parameter was calculated. The system suitability data are stated in Table 1 and the results obtained were within the acceptable range with %RSD of these values should be ≤2.System suitability tests established that the chromatographic system was adequate for the analysis of TH in NSF.

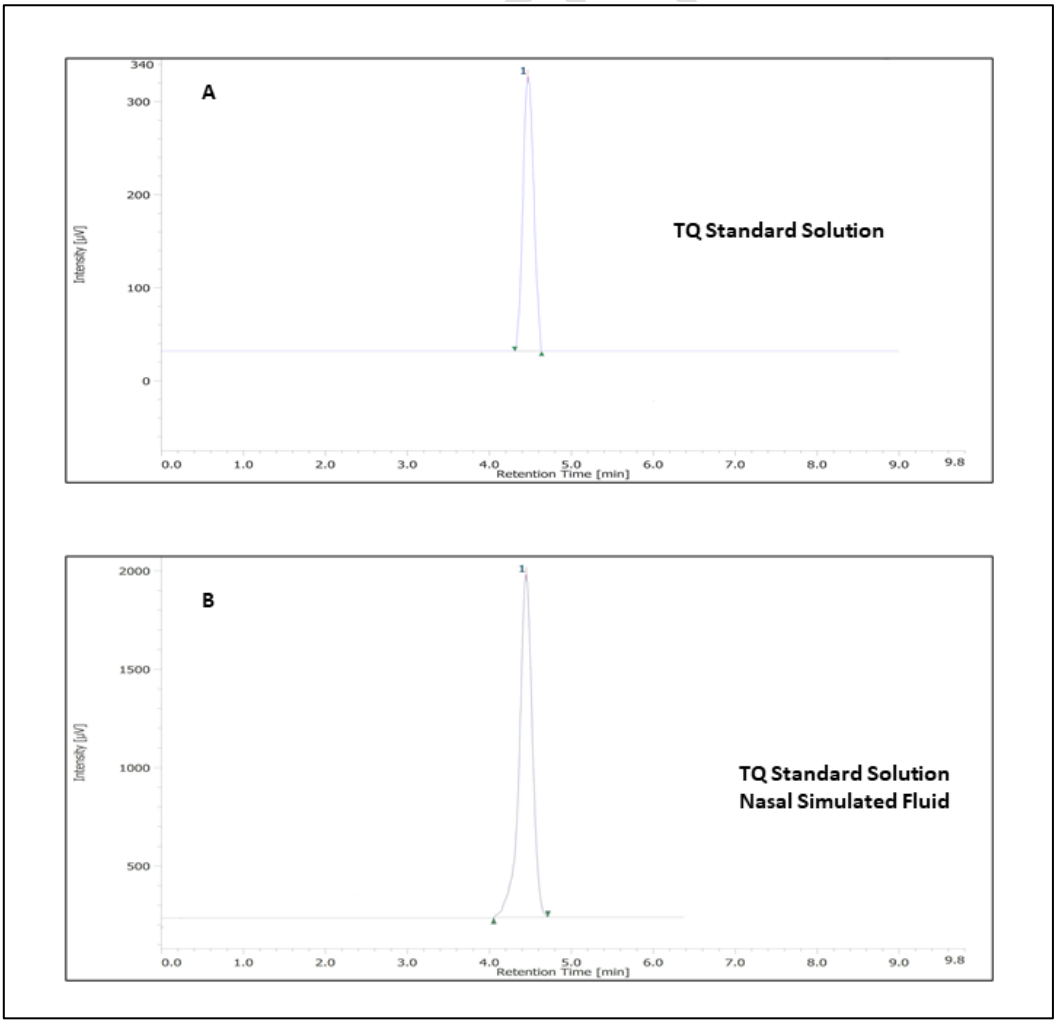

Figure 2: HPLC chromatogram of 20-µL injection of TH in acetonitrile: water (65:35) (A) and acetonitrile: NSF (60:40) (B) respectively.

Table 1: System suitability parameters of TH in acetonitrile: water (65:35) and acetonitrile: NSF (60:40).

Parameter	Acetonitrile: Water (65:35)		Acetonitrile: NSF (60:40)	
	Mean ± SD	%RSD	Mean ± SD	%RSD
Rt	4.450±0.06	0.621	4.721±0.103	0.521
TF	1.00±0.02	0.121	1.20±0.03	0.098
TP	4485±93	1.321	4512±81	2.969

Rt: retention time, TF: tailing factor, and TP: theoretical plates; n=3.

4.1 Method Validation

The validation of developed method was done as per the ICH guidelines with the help of parameters like linearity, sensitivity, accuracy, precision and stability of thymoquinone.

4.1.1 Linearity

The assessment of linearity for the developed method was done by plotting calibration curve between peak areas of drug against concentration of the drug. The curve was linear over the range of 2– 14 μg/mL for both the mobile phases. The regression equations of acetonitrile: water (65:35) and acetonitrile: NSF (60:40) was y=1726.9x-481.29 (R^2=0.9991) and y=1725.4x-409.29 (R^2 = 0.9987) respectively.

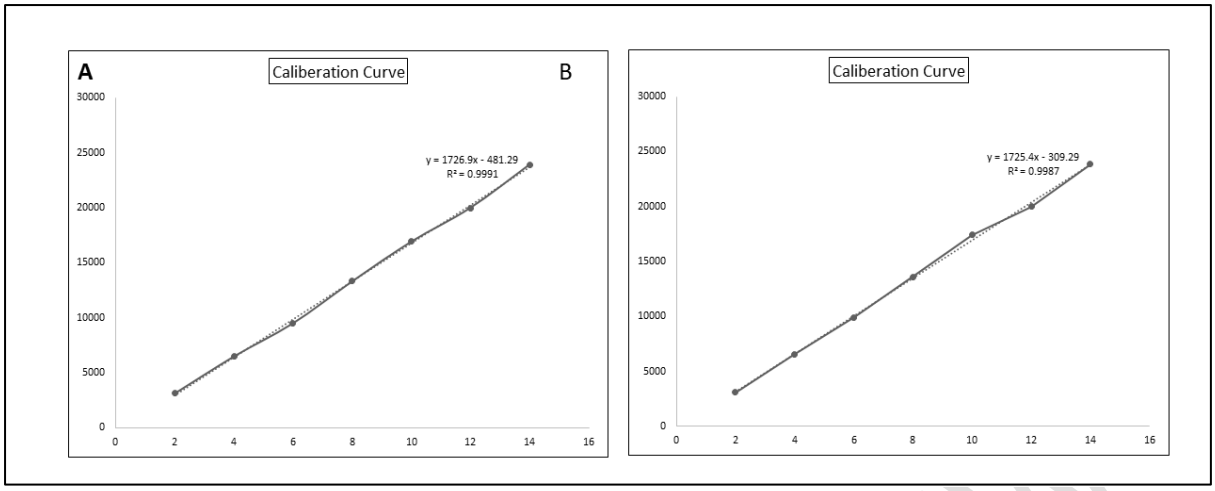

Figure 3: Linearity calibration curves of TH in acetonitrile: water (65:35) (A) and acetonitrile: NSF (60:40) (B) respectively.

4.1.2 Precision and Accuracy

The precision and accuracy of the developed method was checked with by subjecting to repeatability (intra-day) and intermediate precision (inter-day) at three different concentrations for 3 different days in replicates of three and results are shown in table 2, %RSD was found to be less than 2 for all the drugs which indicates that the method is precise. Recovery experiments were done to determine the accuracy of method and results are shown in table 3. The data indicated good accuracy and reproducibility.

Table 2: Intra and Interday accuracy and precision of TH in Nasal Simulated Fluid.

Conc. in µg/ml	Mean ± SD	%RSD
Intra Day (n=3)		
4 µg/ml	99.18±0.312	0.312
8 µg/ml	98.092±0.001	0.012
12 µg/ml	101.081±0.0191	0.0193
Inter Day (n=3)		
4 µg/ml	98.31±0.098	0.099
8 µg/ml	97.08±0.012	0.021

12 µg/ml	97.04±0.112	0.119

Table 3: Recovery study of TH

Conc. Add	Conc. Obt	%Recovery
80% (8 µg/ml)	79.228±0.098	99.05%
100% (10 µg/ml)	98.029±0.021	98.02%
120% (12 µg/ml)	119.021±0.081	99.18%

Conc. Add.: concentration added; Conc. Obt: Concentration obtained; n=3.

4.2 Forced Degradation Study

The stress degradation was done by considering the effect of (thermal degradation) heat, light (photodegradation) and the acidic conditions on the stability of TH. Thymoquinone was degraded up to 28.91% in presence of acid, thermal effect caused 14.80% degradation and 7.955% degradation was seen in incidence of ultraviolet light (UV light) at 24 hrs. The TH exhibited highest stability in NSF with minimum deterioration of 0.79%. The results of stress study indicate that TH was unstable under these conditions. Slight changes in the retention time of TH in case of acidic and photo degradation and increased tailing of the peaks was seen. These degradation peaks were not interfering with the parent peaks. Determination of the degradation products is out of the scope of stated work. The chromatograms of stress conditions are shown in Figure 4 and the percentage degradation of TH is represented in (Table 4).

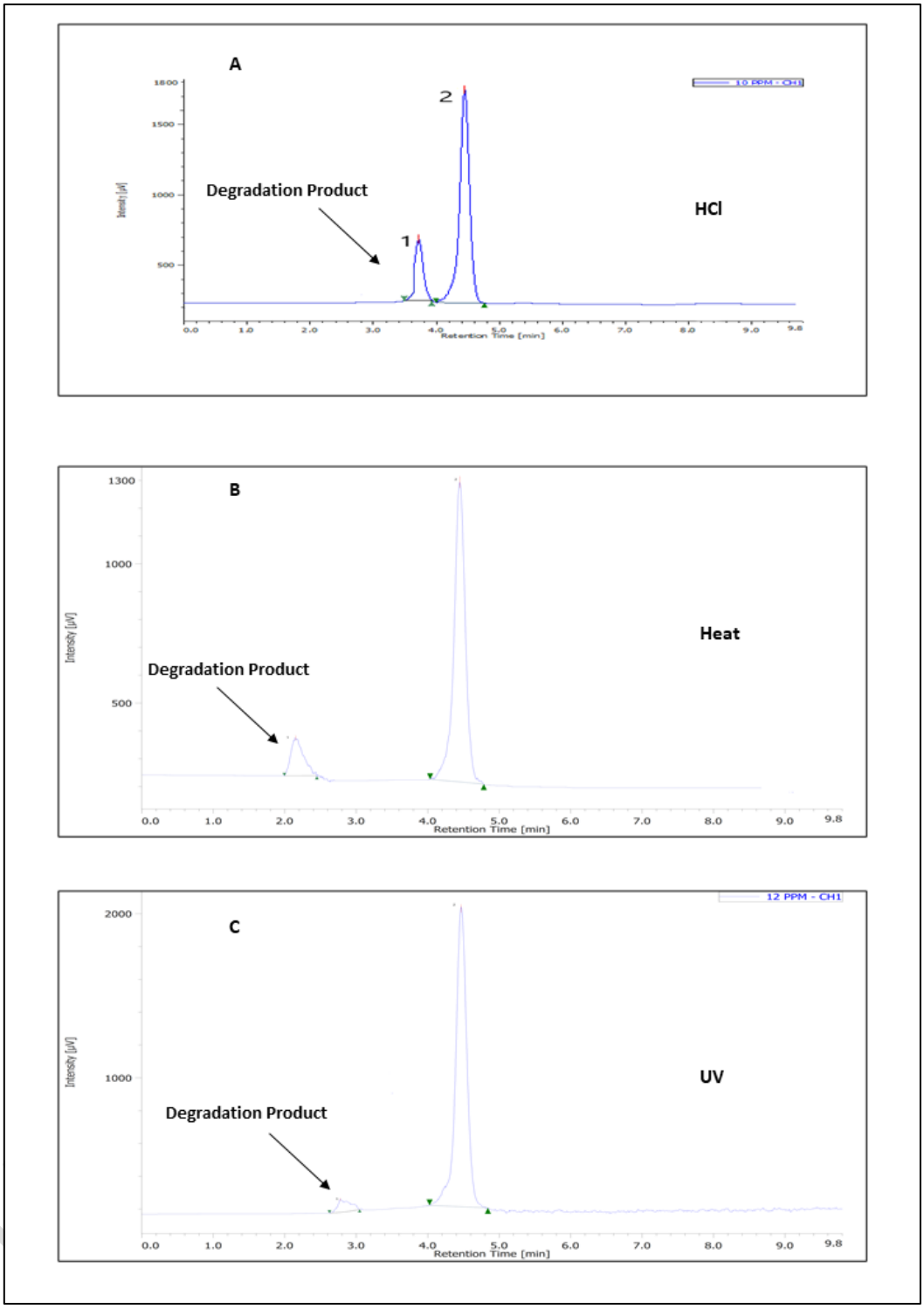

Figure 4: Chromatograms of TH under stress conditions (A) 1 M hydrochloric acid, (B)thermal degradation, (C) photodegradation.

Table 4: Forced degradation studies data of TH in Nasal Simulated Fluid.

Parameter	Acetonitrile: NSF (60:40)	
	% Assay	%Degradation
Nasal Simulated Fluid	99.21±0.012	0.79%
Acid Degradation	71.087±0.197	28.91
Thermal Degradation	85.20±1.972	14.80
Photodegradation	92.045±0.452	7.955

All values are represented n=3

5.0 Conclusion

The developed method shown ideal selectivity and specificity at stated chromatographic conditions in nasal simulated fluid. The recovery studies are found to be >98% and observation of %RSD less than 2 for both intra- and interday measurements indicates a high degree of precision. In the present method, hypersil gold C18 (250x6x5 µm) column has been used at a flow rate of 1 mL/min. The method was optimized with low injection volume. The stability of TH was found to be within the limits indicating that there is no degradation of drugs during the daily analysis and in nasal simulated fluid. This method can be further applied for estimation of TH in NSF for intranasal delivery and various dosage forms.

Statement of Informed Consent: All authors give consent for publication

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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