# A SIMPLE AND VALIDATED HP-TLC METHOD FOR SIMULTANEOUS ANALYSIS OF ETHNO-MEDICINE GALLIC ACID AND EUGENOL

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**ABSTRACT**

A new method has been developed and validated for the simultaneous estimation of gallic acid and eugenol for the poly-herbal formulation. The HP-TLC (High Performance Thin Layer Chromatography) method was developed using pre-coated silica gel 60 $F_{254}$ on aluminum plate and the solvent system used was iso propyl alcohol : hexane: ethyl acetate: glacial acetic acid (50:30:30:1 V/V) and scanned at 254 nm wavelength. The $R_f$ value for gallic acid was 0.608±0.041 and 0.752±0.035 for eugenol. The range of linearity detected for gallic acid and eugenol was 2-10 ng $\mu L^{-1}$. The method was validated in accordance with ICH (International Council for Harmonization) guidelines for linearity, range, LOD, LOQ, precision, specificity and robustness. The developed method was found to be easy, simple, selective, specific and robust.

**Keywords:** High Performance-Thin Layer Chromatography, gallic acid, eugenol, International Council for Harmonization

## ABBREVIATIONS

V/V: volume by volume; nm: nanometer; ng: nanogram; ICH: International Council for Harmonization; LOD: Limit of Detection; LOQ: Limit of Quantification; mg: milligram; mL: milliliter; cm: centimeter; $^0$C: degree Celsius; mm: millimeter; mins: minutes; µL $s^{-1}$: microliter per second; SD: Standard deviation; %RSD: Percentage Relative standard deviation; AUC: Area under the Curve; mM: miliMolar

## INTRODUCTION

Quality, safety and efficacy are the requirements for the herbal medicinal products. Herbs are exposed to various environmental factors which make these herbs prone to variations in their quality and efficacy, which in turn hamper the safety of these herbs as well as herbal products[1].

Worldwide, there's an increase in popularity and demand of herbs and herbal products. But the increase in usage of herbs has brought to light many adverse events which are associated with the poor quality of raw herbal materials and finished products. So, to overcome these adverse events, the tools of modern analytical techniques are being used to standardize the materials and to regulate the quality of the herbs in accordance with the quality aspects[2].

High performance liquid chromatography (HPLC), gas chromatography (GC), mass spectroscopy (MS) and high performance thin layer chromatography (HP-TLC) are popular modern analytical tools for the quality control of raw materials. Whereas in the case of the herbs, HP-TLC analytical tool is considered as the most potent tool due to the simplicity and reliability of technique. Identification, fingerprinting, authentication and quality control of the herbs and herbal products can be done by the HP-TLC technique[3].

Gallic acid (3, 4, 5 - trihyroxybenzoic acid) belongs to the poly-phenolic compound class, and is available in nature in fruits and plants. In pharmaceuticals and other chemical fields, gallic acid is one among the widely used active substances. Gallic acid finds application broadly in cosmeceuticals specifically related to the skin and hair[4]. Anti-oxidant, anti-microbial, anti-inflammatory and anti-cancer are the bioactivities exhibited by gallic acid

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as it's available in most of the plants as a secondary metabolite[5].

Eugenol is chemically known as hydroxyphenyl propene and exists in a nature as an essential oil. Eugenol belongs to Lamiaceae, Lauraceae and Myristicacea families. Eugenol can be considered as an ethno-herb used for decades together due to its potent anti-inflammatory, anti-oxidant and anti-microbial effect. Eugenol oil finds wide application as flavoring agent in foods and cosmetics[6, 7].

## MATERIALS AND METHODS

**Materials/Chemicals:** Marker compound gallic acid was procured from Loba Chemicals and eugenol from HiMedia. All the other reagents used were of analytical grade.

## METHOD DEVELOPMENT

**Preparation of standard solutions**: The standards were prepared by weighing accurately 10 mg in 10 mL of methanol and further dilutions were made accordingly.

### Preparation of sample solution

500 mg of the prepared formulations was weighed and added to 10 mL of methanol, sonicated on bath sonicator for 10 mins, filtered and used.

### Selection of wavelength

The wavelength selected for the scanning of the standards and sample was 254 nm.

### Mobile phase optimization

Many trials were carried out using different mobile phases with different ratios of solvents and the one which gave best elution was selected for the method development and validated[8, 9].

Isopropyl alcohol: *N*-hexane: ethyl acetate: glacial acetic acid (V/V)

10 : 6 : 6 : 0.1

### Optimized chromatographic conditions

HP-TLC chromatography was performed on aluminum backed silica gel 60 $F_{254}$ plate with dimensions of 20X10 cm. Samples were applied with 8 mm band by the CAMAG Linomat V and a total of 15 tracks were applied on the plate. Ascending development was carried out after application for the development of plate in Twin trough chamber and temperature maintained was 25±2 $^0$C. The twin trough chamber was kept for saturation of 10 mins and later the solvent run on the plate was for 70 mm distance and the time consumed was 12-15 mins. The plate was dried at room temperature. The plate was scanned with CAMAG TLC scanner 4 with visionCATS software[10, 11]. Optimized conditions for the method development are given in Table I. The HP-TLC Plate image under UV chamber is shown in Fig. 1.

### Validation

**Linearity:** The stock solution of both the analytes was prepared with different concentrations by using methanol as solvent. Stock solution of 1 mg mL$^{-1}$ was prepared separately for both the analytes and 2 ng µL$^{-1}$ to 10 ng µL$^{-1}$ further dilutions were made using the solvent and then applied on the plate and the data was collected.

**Range:** With results obtained from the linearity, the range of the analytes were determined.

**LOD (Limit of detection):** Statistical principles were applied to the values of the obtained data for the calculation of the LOD (limit of detection) and the data was reported.

**LOQ (Limit of quantification):** Statistical principles were applied to the values of the obtained data for the calculation of the LOQ (limit of quantification) and the data was reported.

**Precision:** The standards of concentration 6 ng µL$^{-1}$ of both the analysis were applied on the plates as six replicates and the plates were allowed to scan and the data was collected and further the inter-day and intra-day precision was carried out with 6 ng µL$^{-1}$.

**Accuracy:** The accuracy of both the analytes was performed by applying triplicates of sample with standard 80 %, 100 %, and 120 % for spiking.

**Specificity:** Specificity parameter was performed by applying tracks with standards solutions, mobile phase and solvent system on the same plate were allowed to develop and scanned for the chromatograms.

**Robustness:** Robustness was performed by deliberately making small changes in composition of mobile phase (±0.1 mL), and the saturation time of the tank and plate after application was varied (±5 mins)[12-14].

## RESULTS AND DISCUSSION

### Method development

### Optimized method conditions:

**Table I: Optimized condition for HP-TLC method development**

| Parameter | Chromatographic conditions |
|---|---|
| **Development chamber** | CAMAG Twin trough chambers |
| **Stationary phase** | Silica gel G 60 $F_{254}$ pre-coated on aluminum |
| **Mobile phase** | Isopropyl alcohol: hexane: ethyl acetate: glacial acetic acid (V/V) 10 : 6 : 6 : 0.1 |
| **Chamber saturation** | 15 minutes |
| **Sample applicator** | CAMAG Linomat V |
| **Application rate** | 0.1µL $s^{-1}$ |
| **Development distance** | 70 mm |
| **Drying of plate** | Room temperature |
| **Densitometry scanner** | CAMAG TLC Scanner 4 |
| **Lamp** | Deuterium |

**Rf values:** The average $R_f$ values for gallic acid was found to be 0.608±0.041 and for eugenol 0.752±0.035. The chromatograms for gallic acid and eugenol are shown in Fig. 2.

### Method validation

**Linearity**: The linearity parameter is shown for gallic acid and eugenol in Fig. 3 and Fig. 4, respectively.

**Range:** The linearity was found within the 2-10 ng $\mu L^{-1}$ and 2-10 ng $\mu L^{-1}$ was considered the range for both gallic acid and eugenol.

### LOD (Limit of detection)

The LOD was calculated from the linearity parameter by using slope and standard deviation. The values of LOD are given in Table II.

### LOQ (Limit of quantification)

The LOD was calculated from the linearity parameter by using slope and standard deviation. The values of LOQ are given in Table II.

**Table II: LOD and LOQ for standards**

| Standard | LOD | LOQ |
|---|---|---|
| Gallic acid | 7.852931 ng $mL^{-1}$ | 23.79676 ng $mL^{-1}$ |
| Eugenol | 8.778257 ng $mL^{-1}$ | 26.60078 ng $mL^{-1}$ |

### Precision

**Precision repeatability:** Repeatability parameter was performed by applying six replicates of both the standards. Intra-day precision and Inter-day precision of gallic acid and eugenol was found to be in the criteria of less than 2% RSD, as shown in Tables III, IV, V, VI and VII.

**Table III: Precision repeatability of gallic acid and eugenol**

| | Gallic acid | | Eugenol | |
|---|---|---|---|---|
| | Rf | Peak area | Rf | Peak area |
| 1 | 0.61 | 0.02912 | 0.752 | 0.02968 |
| 2 | 0.61 | 0.02865 | 0.752 | 0.03001 |
| 3 | 0.61 | 0.02908 | 0.75 | 0.03024 |
| 4 | 0.61 | 0.02836 | 0.747 | 0.0302 |
| 5 | 0.608 | 0.02897 | 0.747 | 0.02989 |
| 6 | 0.608 | 0.03007 | 0.747 | 0.02995 |
| Mean | 0.6178333 | 0.029041667 | 0.749166667 | 0.029995 |
| SD | 0.223803858 | 0.000530453 | 0.002266912 | 0.000189 |
| %RSD | 0.432192837 | 1.826524513 | 0.302591113 | 0.630434 |

**Table IV: Intra-day precision data of gallic acid**

| | Peak area 1 | Peak area 2 | Peak area 3 |
|---|---|---|---|
| 1 | 0.02824 | 0.02908 | 0.02865 |
| 2 | 0.02836 | 0.02897 | 0.02908 |
| 3 | 0.02865 | 0.02897 | 0.02912 |
| Mean | 0.028416667 | 0.029006667 | 0.02895 |
| SD | 0.000210792 | 5.18545E-05 | 0.000260576 |
| %RSD | 0.741790581 | 0.178767515 | 0.900090792 |

**Table V: Intra-day precision data of eugenol**

| | Peak area 1 | Peak area 2 | Peak area 3 |
|---|---|---|---|
| 1 | 0.03074 | 0.03001 | 0.02995 |
| 2 | 0.0304 | 0.0302 | 0.0304 |
| 3 | 0.03079 | 0.03074 | 0.02968 |
| Mean | 0.03064333 | 0.030316667 | 0.03001 |
| SD | 0.00021221 | 0.000378726 | 0.00036373 |
| %RSD | 0.69251796 | 1.249233428 | 1.21203155 |

**Table VI: Inter-day precision data of gallic acid**

| | Peak area 1 | Peak area 2 | Peak area 3 |
|---|---|---|---|
| 1 | 0.02865 | 0.02836 | 0.02912 |
| 2 | 0.02897 | 0.02897 | 0.02908 |
| 3 | 0.02912 | 0.02865 | 0.02897 |
| Mean | 0.028913333 | 0.02866 | 0.029056667 |
| SD | 0.000240069 | 0.000305123 | 7.76745E-05 |
| %RSD | 0.830307013 | 1.064629889 | 0.267320872 |

**Table VII: Inter-day precision data of eugenol**

| | Peak area 1 | Peak area 2 | Peak area 3 |
|---|---|---|---|
| 1 | 0.03079 | 0.0302 | 0.02995 |
| 2 | 0.03074 | 0.03024 | 0.02968 |
| 3 | 0.0304 | 0.03001 | 0.02989 |
| Mean | 0.03064333 | 0.03015 | 0.02984 |
| SD | 0.00021221 | 0.000122882 | 0.00014177 |
| %RSD | 0.69251796 | 0.407569013 | 0.47511551 |

**Accuracy:** The accuracy parameter was carried out for both the standards and the data collected and calculated is shown in Table VIII.

## Plate Images

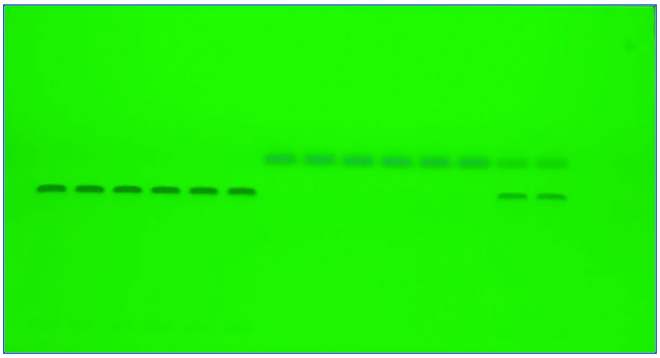

**Fig. 1: Plate image after development under UV chamber**

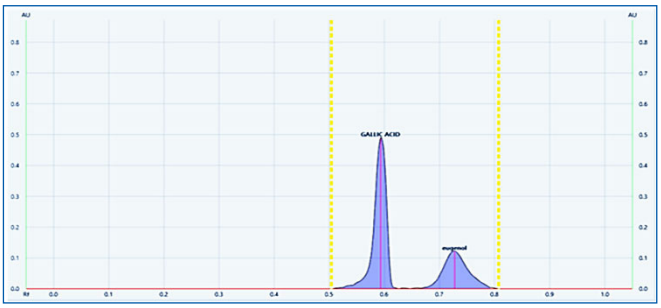

**Fig. 2: Chromatograms of gallic acid and eugenol**

## Specificity

Tracks 1-3 Gallic acid; Tracks 4-6 Eugenol; Tracks 7-9 sample; Tracks 10-12 methanol; Tracks 13-15

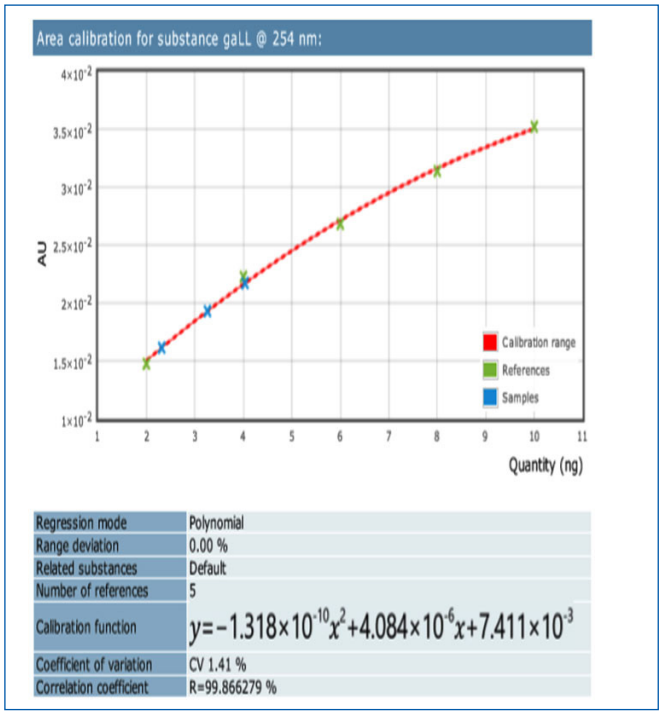


**Fig. 3: Area calibration of gallic acid**

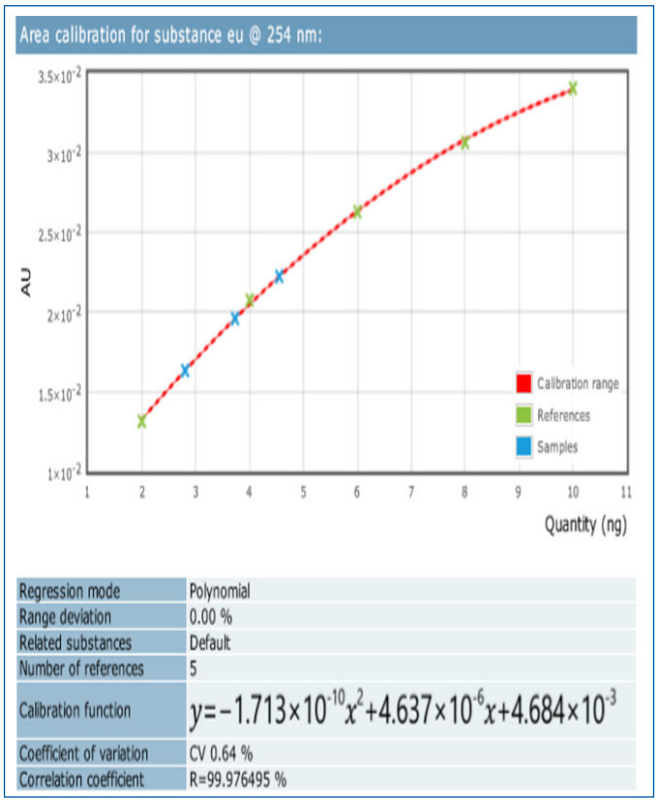


**Fig. 4: Area calibration of eugenol**

**Table VIII: Accuracy data of gallic acid and eugenol**

| Level | Gallic acid | | | Eugenol | | |
|---|---|---|---|---|---|---|
| | AUC of standard and formulation on different spots | AUC of standard and formulation on same spots | Percentage recovery | AUC of standard and formulation on different spots | AUC of standard and formulation on same spots | Percentage recovery |
| 80 | 0.03107 | 0.03046 | 98.05% | 2918 | 2901 | 99.41% |
| 100 | 0.03257 | 0.03226 | 99.04% | 3088 | 3044 | 98.57% |
| 120 | 0.03507 | 0.03458 | 98.60% | 3307 | 3244 | 98.09% |

**AUC—Area under the curve*

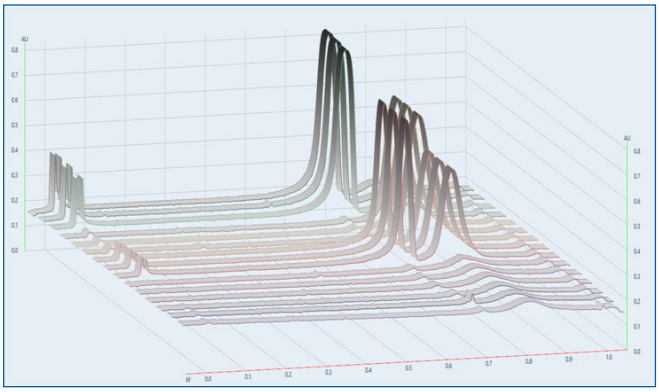

**Fig. 5: Specificity parameter**

*Track 1-3 Gallic acid; Track 4-6 Eugenol; Track 7-9 Sample; Track 10-12 methanol; Track 13-15 Solvent system*

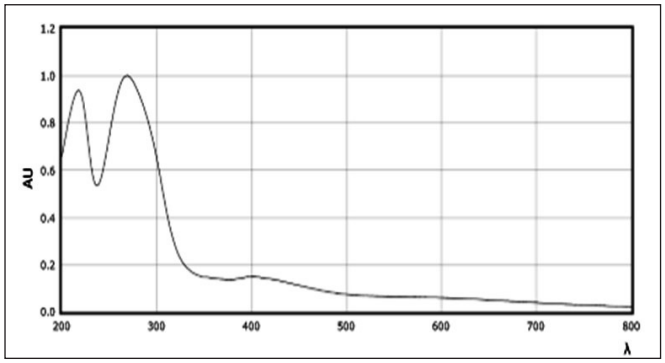


**Fig. 6: Gallic acid spectrum**

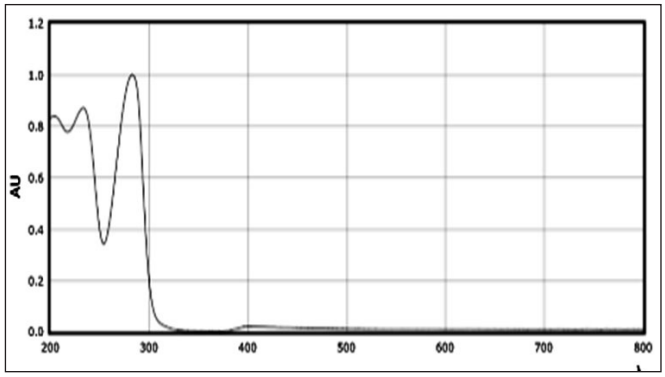


**Fig. 7: Eugenol spectrum**

solvent system; The tracks 10-15 show no peaks, which confirms that the mobile phase and the solvent system do not interfere with the analysis of the standards and the developed method is specific to the standards. The peaks are shown in Fig. 5.

## Robustness

The slight variation in mobile phase composition as well as the saturation time did not show much variation on the method developed and the variations were within 2% RSD.

**Spectra:** The spectra for gallic acid and eugenol are shown in Fig. 6 and Fig. 7, respectively.

## CONCLUSION

A simple and easy HP-TLC method was developed for simultaneous determination of gallic acid and eugenol in various extracts. The developed method was found to be specific, precise, robust and accurate after validation of the developed method with ICH guidelines. Besides, the developed method can be used for simultaneous gallic acid and eugenol determination.

## REFERENCES


1. Steinhoff B.: Quality of herbal medicinal products: State of the art of purity assessment. **Phytomedicine**, 2019, 60, 153003, 1-3.
2. Zhang J., Wider B., Shang H., Li X. and Ernst E.: Quality of herbal medicines: challenges and solutions. **Complement. Ther. Med.,** 2012, 20(1-2), 100-106.
3. Ram M., Abdin M. Z., Khan M. A. and Jha P.: HPTLC fingerprint analysis: a quality control for authentication of herbal phytochemicals, In: High-performance thin-layer chromatography (HPTLC), Springer Publ. (Heidelberg), 2011, pp. 105-116.
4. Choubey S., Varughese L. R., Kumar V. and Beniwal V.: Medicinal importance of gallic acid and its ester derivatives: a patent review. **Pharm. Pat. Anal.**, 2015, 4(4), 305-315.

5. Fernandes F. H. and Salgado H. R.: Gallic acid: review of the methods of determination and quantification. **Crit. Rev. Anal. Chem.**, 2016, 46(3), 257-265.
6. Marchese A., Barbieri R., Coppo E., Orhan I. E., Daglia M., Nabavi S. F., Izadi M., Abdollahi M., Nabavi S. M. and Ajami M.: Antimicrobial activity of eugenol and essential oils containing eugenol: A mechanistic viewpoint. **Crit. Rev. Microbiol.**, 2017, 43(6), 668-689.
7. Khalil A. A., ur Rahman U., Khan M. R., Sahar A., Mehmood T. and Khan M.: Essential oil eugenol: sources, extraction techniques and nutraceutical perspectives. **RSC Adv.**, 2017, 7(52), 32669-32681.
8. Sankar S. S., Sundar S. and De Britto A. J.: L HPLC And HPTLC Fingerprint Profile of *Elytraria imbricata* (Vahl) Pers, **Sciencia Acta Xaveriana,** 2018, 9(2), 49-54.
9. Pundarikakshudu K., Sharma A. K., Bhatt C. J. and Kanaki N. S.: Development and Validation of a High-Performance Thin-Layer Chromatographic (HPTLC) Method for Simultaneous Quantification of Reserpine, Atropine, and Piperine in Sarpagandha Ghanvati, a Classical Ayurvedic Preparation. **J AOAC Int.,** 2019, 102(4), 1021-1026.
10. Abhimanyu K. K., Ravindra C. S. and Avanapu R. S.: A validated HPTLC method for the quantification of friedelin in *Putranjiva roxburghii* Wall extracts and in polyherbal formulations. **Bull. Fac. Pharm. Cairo Univ.**, 2017, 55(1), 79-84.
11. Laila O., Murtaza I., Abdin M. Z., Ahmad S. and Khan M. S.: Development and validation of a high-performance thin-layer chromatography based method for the quantification of trigonelline in fenugreek (*Trigonella foenum*-graecum) seeds, **J. Planar Chromatogr. Modern TLC.**, 2019, 32(2), 95-102.
12. Tomar V., Beeurle T. and Sircar D.: A validated HPTLC method for the simultaneous quantifications of three phenolic acids and three with anolides from *Withania somnifera* plants and its herbal products, **J. Chromatogr. B.**, 2019, 1124, 154-160.
13. Alam P., Kamal Y. T., Alqasoumi S. I, Foudah A. I., Alqarni M. H. and Yusufoglu H. S.: HPTLC method for simultaneous determination of ascorbic acid and gallic acid biomarker from freeze dry pomegranate juice and herbal formulation, **Saudi Pharm. J.,** 2019, 27(7), 975-980.
14. ICH, Q2 (R1), Validation of Analytical procedure, text and methodology, ICH Harmonized Tripartite Guidelines adapted Nov 2005.