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RP-HPLC Method Development, Validation and Application for Quantification Assay and Release Kinetics of Etonogestrel from Contraceptive Subdermal Implant

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Abstract

Etonogestrel (ENG), a biologically active metabolite of desogestrel and a synthetic progestin, is administered via a single-rod implant that represents one of the most effective long-acting reversible contraceptives (LARCs) with minimal side effects. It was aimed to develop and validate a robust method for quantifying ENG and to evaluate its release kinetics from an ENG-releasing single-rod LARC implant, Implanon, using a reversed-phase high-performance liquid chromatography (RP-HPLC) technique. An RP-HPLC method was developed for the quantitative analysis of ENG using Waters C_{18} column (250 × 4.6 mm, 5 µm) where the mobile phase consisted of water and acetonitrile (50:50, v/v), with a flow rate of 1.0 mL/min at room temperature. The injection volume was 50 µl and detection was carried out at a wavelength of 250 nm. All validation parameters of the developed method met the acceptance criteria in accordance with ICH Q2(R1) USP guidelines. The total chromatographic run time was 12 min, with ENG eluting at retention time of 5.5 (± 0.03) min. The method showed linearity over the concentration of 80-120% with a correlation coefficient (r^2>0.998). The limits of detection (LOD) and quantification (LOQ) were 0.08 µg/mL and 0.24 µg/mL, respectively. Accuracy values ranged between 99% and 101%, while precision studies demonstrated %RSD values of <2%. The method remained robust under deliberate variations in chromatographic parameters. In vitro release study revealed an initial release of ENG, followed by a gradual decline consistent with sustained diffusion-controlled release. The release profile showed the best fit to the Higuchi model (R^2>0.98), suggesting diffusion as the primary release mechanism. The validated method is suitable for routine quality control, stability assessment, and release profiling of ENG-containing implants.

Key words: Etonogestrel, contraceptive implant, RP-HPLC, method development and validation, Implanon.

Introduction

At present, different hormonal contraceptive drug delivery systems, including oral contraceptives, injectables, vaginal rings, subdermal implants, and intrauterine device systems or IUDs, are available worldwide for women's use (Maddox *et al.*, 2008). There are two commercial categories of long-acting reversible contraceptive (LARC) implants, one is the double rod containing levonorgestrel (LNG) implant (e.g., Jadelle and Sino-Implant II) and another type belongs to single rod containing etonogestrel (ENG) implant (e.g., Implanon), effective for up to 3-5 years of contraception (Dalaty *et al.*, 2022). Among them, ENG releasing implant is one of the most effective, popular, and acceptable subcutaneous dosage forms as a hormonal contraceptive for the consumers, where a small matchstick-like rod is kept under the upper arm of the female, giving three years of

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contraception for preventing unwanted pregnancies (Implanon *et al.,* 2024).

ENG is a biologically active metabolite of desogestrel, which is a synthetic progestin and binds with progesterone receptors with high affinity in the target organs and stops fertility by impeding one of the important reproductive hormones for ovulation known as luteinizing hormone (LH). It also prevents the passage of spermatozoa by increasing the viscosity of cervical mucus, leading to changes in the uterus lining and finally stops the embedding of a fertilized ovum into the endometrium. In addition, it is quickly absorbed into the systemic circulation after the implant is inserted subdermally, which is almost fully available in the blood. The rate of drug release is 60-70 mcg per day for the first six weeks of ENG implant insertion, then at the end of the first year, its release progressively reduces daily from 35-45 mcg and the drug release is reduced to 35-45 mcg per day by the end of the second year of its insertion, and the rate declines to around 25-30 mcg per day by the end of the third year of implantation. ENG is vastly bound with serum proteins, mainly albumin and also with sex hormone-binding globulin. Once in the bloodstream, it is available; cytochrome P450 (CYP) 3A4 isoenzymes in the liver metabolizes ENG (Maddox *et al.,* 2008; Implanon *et al.,* 2024).

ENG is a chemical analogue of 19-nortestosyerone with molecular weight of 324.6 and its chemical structure ($C_{22}H_{28}O_2$) is shown in the Figure 1. Various analytical techniques such as UV, (Berzas *et al.,* 1997). RP-HPLC, (Pal *et al.,* 2020; Rajeswari *et al.,* 2019). Mass spectroscopy and LC-MS/MS (Diaz-Cruz *et al.*, 2003) etc. can be used for quantification of LNG and ENG, out of which HPLC is one of the vital methods because of its linearity, accuracy, specificity, precision, and robustness (Veeran *et al*., 2021).

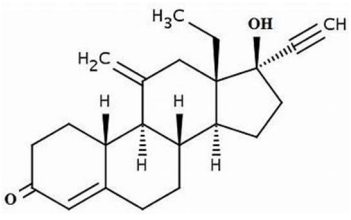

Figure 1. Chemical structure of ENG.

Despite available analytical reports, limited studies have focused on developing a validated stability-indicating RP-HPLC method for simultaneous assay and release evaluation of ENG from subdermal implant systems. Since method development and validation of analytes performs a very significant role in the drug development in pharmaceuticals, we had an effort to develop and validate a very easy, simple and constant RP-HPLC method as per ICH and USP guidelines (Fuchs *et al.,* 2020; IIegbusi *et al.,*; Wathoni *et al*., 2018) to determine ENG from LARCs subdermal hormonal implants in the current report.

Materials and Methods

Chemicals and instrumentation with RP-HPLC: ENG was collected (active pharmaceutical ingredient) from Techno Drugs Ltd., Bangladesh, and purchased Implanon, a single rod progestin-releasing subdermal implant system containing 68 mg of ENG with 4 cm in length and 2 cm in diameter, labeled for three years of contraception from the supplier. Acetonitrile (Fischer-Scientific, UK) and chloroform (Merck, Germany) as HPLC-grade solvents and HPLC-ready water were used from the Ultra-Pure System. Moreover, 0.45 µm membrane disc filters and 0.22 µm- syringe filters, electronic micro balance, a sonicator and a planetary centrifugal mixer were used. The Waters RP-HPLC system, equipped with a degasser, autosampler, quaternary solvent delivery pump, column oven and UV/UV-VIS detector, was used for the analysis and Empower Software was used for validation of the method.

Wavelength detection: 0.1 mg/ml of ENG stock solution was prepared with acetonitrile (60%) and serial dilution was performed to make the final concentration of ENG (10 µg/ml). This solution was then scanned between 200 nm and 400 nm using a UV spectrophotometer at a 250 nm wavelength and the spectra were recorded.

Chromatographic condition: All the analysis were carried out using the Waters RP-HPLC system. The ENG analysis was performed with the help of a C_{18} column of 250 × 4.6 mm dimensions, 5 µm-sized particles in an isocratic mode of mobile phase

composed of water and acetonitrile in the ratio of 50:50 (v/v). In addition, ENG elusion was conducted at room temperature following one ml per minute flow rate, 50 µl injection volume with total run time of 11 minutes at the detection wavelength of 250 nm. Before injecting blank solution as well as drug solution, the HPLC system was equilibrated for almost one hour by degassing with mobile phase. Then the blank solution was filtered through the syringe filter of 0.22 µm and injected to ensure that there was no interference with the solvent.

Preparation of blank solution, stock solution and standard solution of ENG: The blank solution was prepared with a 60% acetonitrile solution. 10 mg of ENG working standard in powder form was weighed accurately and transferred to an amber colored 100 mL volumetric flask. Then 60 mL of diluent (mobile phase) was added to it and sonicated for 20 minutes for complete dissolution, and the volume was brought up to the mark through vigorous mixing. This solution is termed as standard stock solution. From this stock solution, we performed consequent dilution with the solvent to make the ENG standard solution of 3.0 µg/ml. The prepared solution was then filtered for analysis by HPLC through a syringe filter of 0.22 µm.

Preparation of the calibration curve of the standard solution: ENG standard stock solution was used for the preparation of a calibration curve with different concentrations.

Preparation of the sample solution: We collected some implants (Implanon) with 4 cm in length and 2 cm in diameter and took average weight of 3 implant rods and cut into small fragments (~ 1-mm length) equivalent to one rod were transferred to 100 ml of volumetric flask, 50 ml of reagent grade chloroform was added, sonicated for 20 minutes and allowed to stand for at least one hour, and then diluted with chloroform to 100 ml volume and filtered with Whatman filter paper and 0.5 ml solution was transferred into a 100 ml dried volumetric flask and allowed to evaporate for dryness. The mobile phase (60 ml) was added again and sonicated for 20 minutes to get the final volume. After filtration by a 0.22 µm syringe filter, all the samples were finally analyzed by HPLC following the aforementioned chromatographic parameters.

RP-HPLC method validation: As per ICH guidelines Q2 (R1), we validated the method for parameters such as specificity, linearity, accuracy (recovery), precision, robustness and stability of the sample solution.

System suitability: We *a*ssessed the suitability of the system through the injection of blank solution and standard solution of ENG by measuring some factors like tailing factor, theoretical plates, and relative standard deviation (RSD). The limit of acceptance for system suitability is for ENG (RSD), with no more than 2% of peak area, a USP tailing factor no more than 2 and theoretical plates no less than 2000 for the column.

Specificity: We documented the method specificity by the HPLC system through injecting blank solution, placebo, ENG standard solution, and sample solution. The obtained chromatograms were analyzed so that blank solution and placebo solution showed at the retention time of the drug without any interference.

Linearity: We checked for linearity with a series of solutions by diluting quantitatively the ENG stock solutions ranging from 40% to 120% and plotted a calibration curve with the help of the average peak area of triplicate injections against concentration. Additionally, we measured the slope, intercept, and correlation coefficient to confirm the linearity of the proposed method.

Accuracy: We measured the accuracy of the analytical technique for three spiked concentrations of ENG. For each concentration, triplicates were set and obtained percent recovery and percent RSD in the acceptable limit.

Precision: We spiked six different standard solutions of ENG (3.0 µg/ml) and intra-day precision for repeatability was evaluated for the placebo solution on a similar day, with a small break in time at similar operating factors. The percent recovery and %RSD were measured to check the repeatability, and

inter-day precision was also evaluated by comparing those values for two different days of analysis.

Robustness: We performed the robustness of the method by changing a small difference in the flow rate of ± 0.2 ml/min and the mobile phase composition of ± 0.5%. The standard ENG solution was measured at different conditions, and then the percentage RSD for ENG peak area, number of theoretical plates, and the USP tailing factor was measured.

Stability: The stability of ENG implant systems was tested at regular break for up to 72 hours under room temperature. During each of the intervals, the values for the ENG peak area, theoretical plates, and tailing factor were evaluated.

Applicability of the method: The applicability of the method was evaluated for the assay and release profile study of the marketed formulation of LARC implant.

Assay of ENG implant. In our study, we carried out the assay of the commercially available product Implanon, containing 68 mg of ENG in a single rod, using the developed analytical method by HPLC and quantification of ENG was done for each implant. Each sample was analyzed by using peak area measurement at 250 nm and then the amount of ENG released per implant was calculated.

Release kinetics of ENG: Six implants (Implanon) were taken and suspended in a 100 ml stoppered transparent iodine bottle. The distance to the bottom of the bottle was about one cm. Then 100 ml of purified water was added, and the iodine bottles were placed in a constant temperature oscillator (37°C). The round-trip speed was 60 times per minute. The amplitude was 3 cm. After 24 hours of constant temperature oscillation, the solution was discarded. Then 100 ml of purified water was added and continued oscillation for 24 hours. This operation was repeated for 7 days. The solutions from the 3rd, 5th, and 7th days were collected as the test solution and filtered through a 0.45-micron filter. Standard solution and sample solution were then injected after the system suitability test. Finally, chromatographic analysis was performed, and the amount of ENG released from each implant per day was measured.

Results and Discussion

Method development and optimization by RP-HPLC: For method optimization, we used various ratios of water: acetonitrile as 30:70, 40:60, 50:50 and 60:40. The optimum mobile phase was selected based on retention time, resolution, number of theoretical plates and tailing factor at 250 nm of detection wavelength by UV spectrophotometry. An appropriate separation of ENG having better resolution and less tailing factor of water and acetonitrile solvent as a ratio of 50:50 (v/v) with a flow rate of one mL per minute by an HPLC column under isocratic conditions through 50 µl injection, fixing as an optimized condition.

Method validation by RP-HPLC: The system suitability report presented in our study reflects that all the parameters met the acceptable limit for the ENG standard solution. The drug had a retention time of 5.5 minutes (± 0.03) having percent RSD of 0.1 of peak area. It indicated good repeatability in the system. There was no interference with diluents, impurities, or any other excipients at the detection wavelength. The observed tailing factor was smaller than 1.05, which showed that the highest symmetry was okay. Moreover, the number of theoretical plates was no less than 2000 in all runs of the chromatograph.

Specificity of method: Chromatograms obtained from blank, placebo and standard solution of ENG showed that no interference detected at the retention time of 5.5 (± 0.03) minute for ENG (Figure 2). The peak was obtained for blank solution at 2.5 minute and for placebo at 1.2-minute indicating that the peak found was pure for the analyte that confirmed the specificity of this method with respect to retention time.

Linearity of the method: The calibration curve with standard ENG solution obtained a linear (Figure 3) relationship with regression coefficient (r^2) 0.999 in various concentration ranges, and the results obtained indicated that the method met the limit of acceptance for linearity of the method.

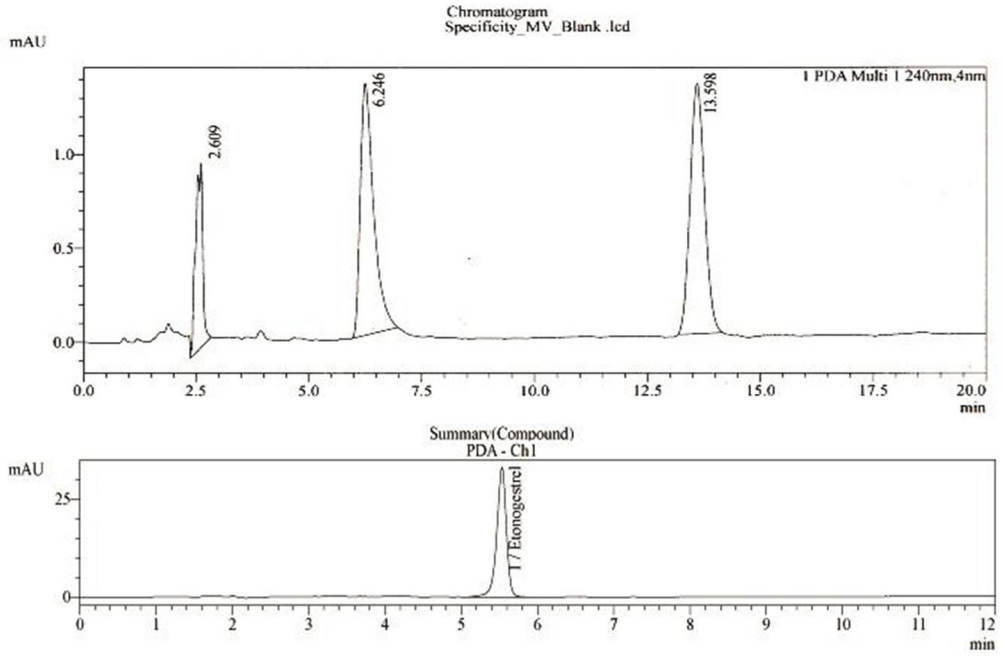

Figure 2. Chromatograms for the method validation of ENG.

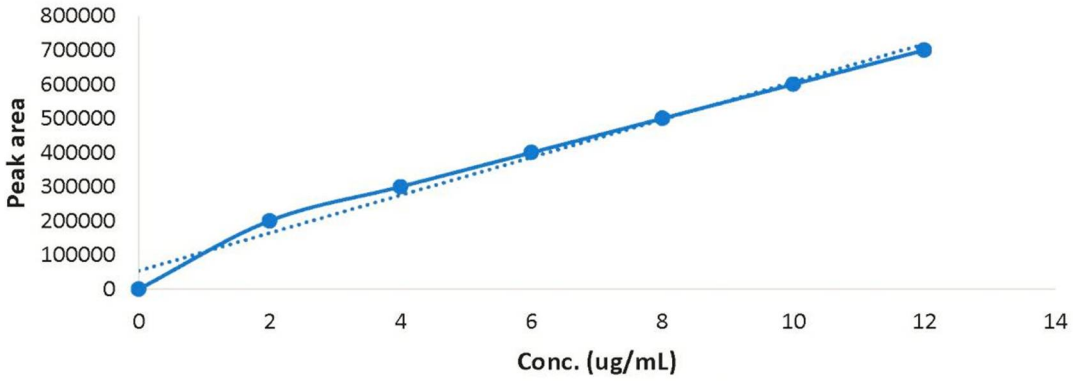

Figure 3. Linearity curve of ENG.

Accuracy of the method: The closeness of the obtained results for three different concentrations at 80, 100 and 120% of ENG solution showed the accuracy of the proposed method. The percent recovery was obtained to be in the range of 99% to 100% from 3 spiked samples and the mean percent recovery of ENG at three various concentrations met the acceptance criteria shown in table 1 and hence the method was accurate.

Precision of the method: The average RSD (%) for ENG in the repeatability study of six replicate injections is shown in the table, and the values found for intra-day as well as inter-day quantification were within the limit, confirming high precision as well as repeatability of the developed method, displayed in table 2.

Table 1. Study of accuracy and recovery.

Level (%)	Spiked conc. (µg/ml)	Obtained conc. (µg/ml)	Recovery (%)	RSD (%)
80	4.5	4.5	99.5	0.30
100	9.5	10.5	100	0.50
120	13.5	13.8	99.9	0.55

Table 2. Intra-day and inter-day results of precision.

Precision	Peak area	% RSD	% Recovery factor	% RSD Recovery
Intra-day (repeatability)	67740	0.660	1.326	0.50
Inter-day				
Day 1, System 1, Analyst 1	65650	2.654	1.554	0.45
Day 2, System 1, Analyst 1	65765	2.542	1.597	1.33
Day 2, System 2, Analyst 2	66765	2.753	1.469	2.50

Robustness of the method: Robustness was studied in three sample solutions of ENG by making small changes in chromatographic conditions, and the values found are presented in table 3. The percent assay of three ENG standard solutions met the limit of acceptance (tailing factor was less than 2 and the percent RSD was less than 2%) approving that the method is robust with respect to small changes in flow rate of mobile phase (± 0.1 ml/min), wavelength (± 1 nm) and volume of mobile phase composition (± 5%).

Table 3. Results of robustness under various parameters.

Parameters	Percent RSD	Tailing factor	Theoretical plate
Flow rate at 1.3 ml/min	0.10	1.625	36923
Flow rate at 0.7 ml/min	0.09	1.554	35883
Water: Acetonitrile, 40:60	0.08	1.597	37976
Water: Acetonitrile, 20:80	0.07	1.469	34645

Table 4. Results for the stability study from ENG implant.

Time (hours)	Peak area	Tailing factor	Theoretical plate
0	76750	1.625	43923
24	77650	1.774	44883
48	79765	1.737	44976
72	71765	1.762	45645

Stability study: The analyte stability was evaluated by analyzing the ENG standard solution and sample solution at various time intervals by keeping them at room temperature and the obtained results found to be stable for 72 hours. The parameters like peak area, tailing factor and theoretical plates are shown in the table 4 met the acceptance limit and confirmed that there was no significant decomposition of ENG.

Assay of ENG from the implant: The validated method was used for ENG analysis from implants of market preparations (Implanon) containing 68 mg ENG (Figure 4). The mean ENG content was determined to be 97.58% to 102.50% (± 0.04%). The time required for sample preparation was less than half an hour. The total run time was 12 minutes, enabling a quick analysis of several samples in case of routine or quality control analysis of ENG from silicone-based implant rod. The low RSD values indicated the suitability of the proposed method for analysis of ENG releasing subdermal implant drug delivery system.

Release kinetic evaluation of implants: The graph in Figure 6 illustrates the release profile of ENG from Implanon, showing an in vitro dissolution pattern remarkably similar to its mean daily in vivo release. The release rate was approximately 60-70 µg per day during the first six weeks after implant insertion, decreasing to 35-45 µg per day by the end of the first year. The rate remained around 35-45 µg per day at the end of the second year and declined further to approximately 25-30 µg per day by the end of the third year. This analysis revealed no significant differences in release behavior among the ENG formulations, which may explain the lack of distinction observed in the in vivo study outcomes.

Release kinetic analysis: Release data were fitted to commonly applied kinetic models, including Zero-order, First-order, Higuchi *and* Korsmeyer–Peppas. The highest correlation coefficient ($R^2 > 0.98$) was obtained with the Higuchi model, indicating that diffusion through the polymeric matrix governed drug release. The Korsmeyer–Peppas release exponent further supported Fickian diffusion as the primary mechanism.

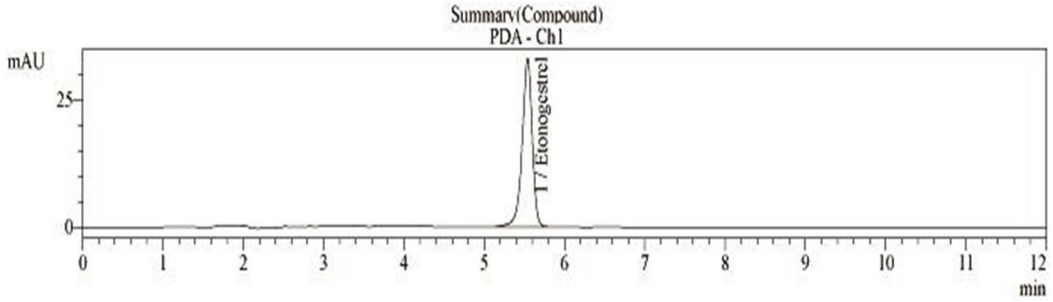

Figure 4. Chromatogram for the assay of ENG from the implant.

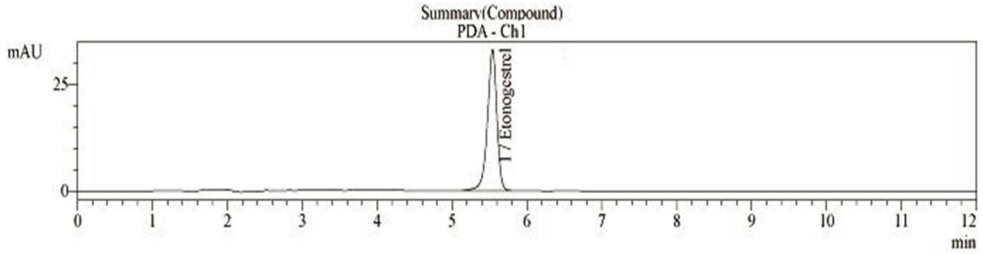

Figure 5. Chromatogram for the release profile of ENG from the implant.

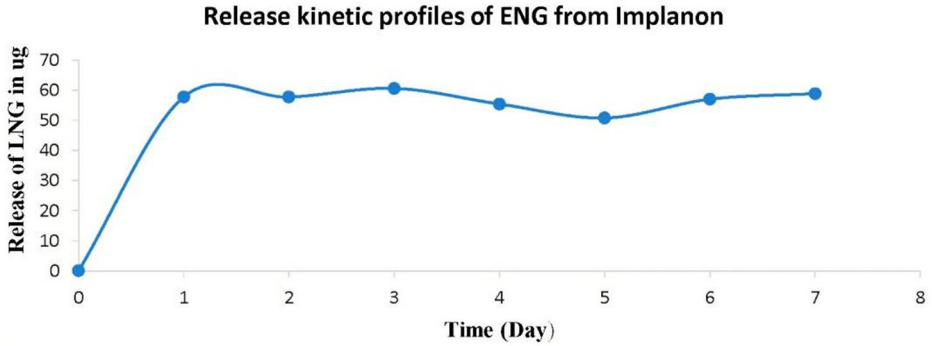

Figure 6. Release kinetic profiles of ENG from the implant.

Conclusion

The method was validated in accordance with ICH guidelines for specificity, linearity, accuracy (recovery), precision, robustness, and stability of the analytical solution, with all results found to be within acceptable limits. Therefore, the validated method was established to be specific, linear, accurate, precise, and robust for the quantification of ENG from implants. Given its simplicity, rapidity, and ease of sample preparation, the developed RP-HPLC method is suitable for routine analysis, process quality control, and stability studies for the determination of ENG in implant formulations. Furthermore, this method can be effectively applied to evaluate the release kinetics of marketed formulations (Implanon) without interference. The short sample preparation time of less than 30 minutes and total chromatographic run time of 11 minutes enable rapid analysis of a large number of samples, making it highly efficient for routine and quality control applications of ENG implants.

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Author Contributions

Conceptualization, AKLK; methodology, AKLK, EH, and SCB; formal analysis, AKLK; MM supervision, EH and SCB; writing-original draft preparation, AKLK; writing-review and editing, AKLK, MM, EH, and SCB. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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