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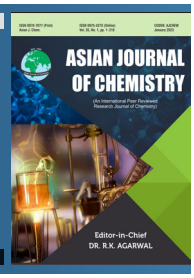


# Liquid Chromatographic-Electron Spray Ionization-Mass Spectroscopy Technique for the Simultaneous Determination of Ethinyl Estradiol & Etonogestrel and Characterization of New Forced Degradation Compounds

J. David Blessing Rani[1,2,*] and C. Asha Deepti[1]

[1]Department of Pharmaceutical Analysis, GITAM School of Pharmacy, GITAM (Deemed to be University), Visakhapatnam-530045, India
[2]School of Pharmacy, Centurion University of Technology and Management, Balasore-756044, India

*Corresponding author: E-mail: ranijakkala@gmail.com

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The aim of the current study was to develop, validate a precise, rapid, accurate liquid chromatography-mass spectrometry (LC-MS) method for the simultaneous estimation of ethinyl estradiol and etonogestrel. The retention times of ethinyl estradiol and etonogestrel were found at 3.112 min and 4.399 min, respectively, whereas the linear range were found in the concentration ranges from 0.04-0.23 µg/mL & 3-18 µg/mL with slope coefficient ($R^2$) of 0.9992 and 0.9991 correspondingly. Similarly, the accuracy values for ethinyl estradiol and etonogestrel were found to be 100.5% and 100.1% correspondingly. As per ICH Q2(R1) guidelines, the method was validated with liquid chromatography-mass spectrometry to confirm the newly obtained degradation compounds chemical structures of ethinyl estradiol and etonogestrel.

**Keywords: Ethinyl estradiol, Etonogestrel, LC-MS, Accuracy.**

## INTRODUCTION

The liquid chromatography-mass spectrometry (LC-MS) analytical chemistry technique has been employed effectively in the drug development processes such as metabolic stability testing, metabolite identification, glycoprotein mapping, drug testing-*in vivo*, impurity detection of impurity, peptide mapping, bio-affinity screening and dereplication of natural products [1]. Ethinyl estradiol (*m.w.* : 296.40; *m.f.* $C_{20}H_{24}O_2$), chemically known as 19-nor-17-pregna-1,3,5(10)-trien-20-yne-3,17-diol is a colourless and crystalline powder, which is readily soluble in alcohol. It falls under the category of contraceptives and generally used in the treatment of androgen-dependent diseases, hirsutism, acne and seborrhea [2]. Gonadotropin suppression is the mechanism by which combination of different contraceptives exert their effect. The gastrointestinal system is responsible for the rapid and almost total absorption of ethinyl estradiol; yet, due to first-pass metabolism in the liver and gut mucosa, the bioavailability of this compound is only between 38 and 48% [3]. A steroid progestin called 11-methylene levonorgestrel, also known as 3-keto-desogestrel or etonogestrel used in hormone contraceptives along with ethinyl estradiol [4]. Etonogestrel (*m.w.* : 324.50; *m.f.* $C_{22}H_{28}O_2$) chemically known as 11-methylene-17α-ethynyl-18-methyl-19-nortestosterone; 11-methylene-17α-ethynyl-18-methylestr-4-en-17β-ol-3-one and generally use to prevent pregnancy [5-10]. Nexplanon and Implanon are two other brands of injectable dosage forms that are administered under the skin in the upper arm. Whereas NuvaRing and Circlet are two brands that sell it as vaginal ring when combined with the estrogen ethinyl estradiol [11].

The literature review conducted on ethinyl estradiol and etonogestrel indicated that only derivative spectroscopic analysis has been reported [12]. However, for ethinyl estradiol with other drugs, derivative spectroscopy [13], UPLC-MS [14-16], for fixed combination dosage form of Nuvaring and with other com-binational drugs on HPLC [17-19] are reported. To the best of our knowledge, there is no published data about the use of HPLC-MS for the analysis of the fixed combination of ethinyl estradiol and etonogestrel. Furthermore, there is a lack of study regarding stability tests and forced degradation studies related to the simultaneous quantification of ethyl estradiol

and etonogestrel. So far, no study has provided a complete validated stability-indicating LC-MS approach for estimating ethinyl estradiol and etonogestrel simultaneously while accounting all known degradation products. Therefore, accurate, quicker and more cost-effective approach for determining ethinyl estradiol and etonogestrel as well as the characterization of results of forced degradation together was still required. The current study aimed to develop a rapid and reproducible LC-MS method for the simultaneous quantification of ethyl estradiol and etonogestrel, in accordance with ICH method validation criteria.

## EXPERIMENTAL

All the HPLC grade solvents were procured from Merck Ltd. India. The drug samples *viz*. thinyl estradiol and etonogestrel were generously gifted by Shree Icon Pharmaceutical Laboratories, Vijayawada, India. For the LC-MS analysis, Alliance HPLC e-2695 (Waters) liquid chromatography combined with Shimadzu-8045 mass spectrometry was used.

**Chromatographic conditions:** HPLC analysis was performed using a Waters Alliance-HPLC system fitted with a 2695 separation module and a 2998 type photo diode array detector, and data was collected using Empower® version 2. For chromatography, an X-bridge phenyl (250 × 4.6 mm, 5 µm) column was employed. Acetonitrile:hexane sulphonic acid and orthophosphoric acid (OPA) buffer (pH 2.5) (40:60 %v/v) were used as mobile phase. Samples tested using a injection volume–10 µL, flow rate–1 mL/min, 6 min runtime and a constant temperature during the process. A photodiode array detector with a 236 nm wavelength was used to detect the drugs.

In forced degradation studies, the operational conditions for mass spectrometry study of ethinyl estradiol and etonogestrel on electrospray ionization (positive) mode were increased. The other conditions were the collision energy–14 V; ion spray voltage– 5500 V, collision gas–ultra pure nitrogen gas, declustering potential–40 V.

**Preparation of buffer:** Hexane sulphonic acid (1.8 g) was dissolved in 1 L water followed the addition of orthophosphoric acid till the buffer solution becomes pH 2.5 and then filtered the solution using 0.45 µ pore size membrane filter paper.

**Mobile phase preparation:** Mixed acetonitrile and buffer in the proportion 40:60 and 0.45 µ pore size, membrane filter paper was used to filter the prepared solution.

**Preparation of working standard solution:** Accurately, weighed 12 mg etonogestrel and 5mg ethinyl estradiol were added to a volumetric flask (50 mL) as working standards. The final volume was make up with diluent and sonicated 30 min to obtain a 100 µg/mL concentration of ethinyl estradiol and 1200 µg/mL etonogestrel stock solution. To obtain 10 µg/mL ethinyl estradiol and 120 µg/mL etonogestrel concentrations, 1 mL of the aforesaid stock solution was pipetted out and added to a 10 mL capacity measuring flask and finally the final volume was adjusted with diluent.

**Preparation of sample solution:** Ten tablets were weighed and then the mean weight was calculated. The one pill was weighed, transferred to a 500 mL graduated flask after being crushed in a mortar and pestle followed by the addition of 60 mL of diluent, sonicated for 30 min and then diluent was added to make the solution. The solution was filtered using a 0.45 syringe filter. Now, 1 mL aliquot of the filtered solution was transferred into a 10 mL volumetric flask and the final volume was adjusted using diluents which yielded a concentration of 10 µg/mL for ethinyl estradiol and 120 µg/mL for etonogestrel.

**Method validation of the optimized method:** The reverse phase high performance liquid chromatography method was validated in accordance with the regulations outlined by the International Council of Harmonization (ICH) Q2 (R1). The validation process involved measuring the precision, accuracy, efficiency, limit of detection (LOD), limit of quantification (LOQ), robustness and linearity of the system.

**System suitability:** To conduct an analysis of the system's performance, an examination was carried out on the suitable characteristics of the system. The chromatogram was obtained by administering six injections of the standard solution, which corresponds to the label claim.

**Specificity:** To detect potential interference caused by characteristic peaks, separate injections of the diluent (blank), placebo, working sample, and standard solution were performed at a concentration of 100 µg/mL.

**Precision:** According to ICH guidelines, the procedure's repeatability and precision were assessed. The chromatograph was obtained with samples from six repeated injections (n = 6) at the homogenous strength (100 µg/mL). Reproducibility and ruggedness were both tested by conducting the tests on the same day and on following days.

**Accuracy:** The accuracy of the suggested technique was assessed by the utilization of recovery trials employing the spiking technique. The pre-analyzed sample was introduced into solutions of the working standard at predefined concentrations (50%, 100% and 150%) in order to conduct recovery experiments. The solutions were prepared in triplicate to ensure precision.

**Linearity:** The linearity of the standard ethinyl estradiol and etonogestrel solutions was assessed across a range of concentrations. A total of six standard solutions were prepared, each with concentrations ranging from 0.04 to 0.23 µg/mL and 3 to 18 µg/mL. These solutions were subsequently injected for analysis. To establish the calibration equation and coefficient of correlation, a linear least-squares regression analysis was employed.

**LOD & LOQ:** The LOD and LOQ for ethinyl estradiol and etonogestrel were calculated with the calibration curve technique. Ethinyl estradiol and etonogestrel solutions were produced and injected (n = 3) within the linearity range. The concentration was plotted against the mean peak areas.

**Robustness:** To assess the robustness of the presented method, tailing factor, theoretical plates and resolution of ethinyl estradiol, etonogestrel peaks were investigated. The impact of a 0.1 mL/min variation in flow rate on the developed approach was evaluated. There was a 10% shift in the mobile phase's composition from the organic phase's starting point. The aqueous portion of mobile phase was controlled in all of the aforementioned setups.

**Forced degradation studies:** Stress trials were conducted using 1.50 ppm and 12 ppm ethinyl estradiol and etonogestrel working standard solutions, respectively, to determine the speci-ficity, stability-indicating property of the proposed method. The stress conditions of exposure of drug to heat (6 h at 105 ºC), acid (1 N HCl - 15 min), alkali (1 N NaOH - 15 min), oxidation (10% $H_2O_2$ for 15 min), reduction (10 % $NaHSO_4$ for 15 min), photolytic (exposed to sunlight for 6 h), water (refluxed for 15 min), were used to attempt the intended degradation. In order to assess the stability of the sample, solutions were introduced into the apparatus and chromatograms were subsequently obtained.

## RESULTS AND DISCUSSION

**Method optimization:** The optimal LC-MS method was developed using trial and error methods. The auto sampler and the analytical column temperature was kept stable at the normal temperature. The retention times of ethinyl estradiol and etonogestrel in the chromatogram were found to be 3.112 and 4.399 min, respectively (Fig. 1). Prominent selectivity and specificity was developed by fine adjustments of variables in the system work. The analysis of peak tailing and the determination of theoretical plate count indicate that all parameters fall within acceptable ranges (Table-1).

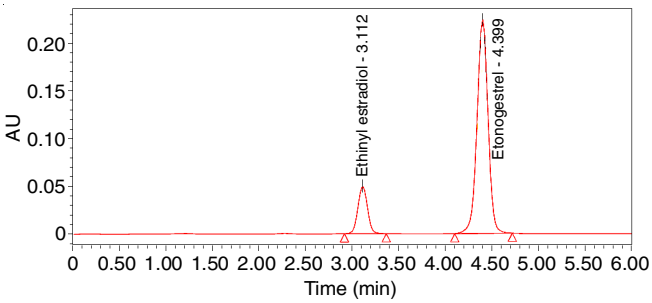


Fig. 1. Optimized chromatogram of ethinyl estradiol and etonogestrel

TABLE-1
SYSTEM SUITABILITY RESULTS

| Variables | Ethinyl estradiol | Etonogestrel |
|---|---|---|
| Plates number | 5634 | 7825 |
| Tailing | 1.11 | 1.05 |
| Resolution | – | 5.28 |
| Elution time of peak | 3.112 | 4.399 |

**Specificity:** There were no instances of co-eluting peaks detected within the retention times of ethinyl estradiol and etonogestrel, suggesting that the peak of the analyte was free of impurities and that the excipients present in the formulation did not cause any interference with the target analyte. The retention period of ethinyl estradiol and etonogestrel was unaffected by the presence of other excipients as depicted in Figs. 2 and 3, respectively.

**Precision:** The study determined that the maximum permissible value for the percent relative standard deviation (RSD) of ethinyl estradiol and etonogestrel is 2%. Consequently, the methodology employed exhibits robustness, replicability and accuracy when applied to a 48 h analysis. Table-2 presents a summary of the findings.

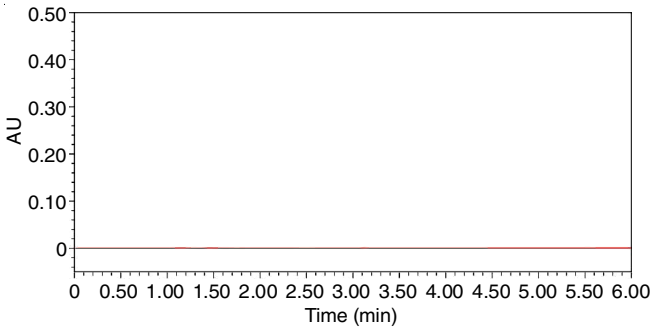


Fig. 2. Blank chromatogram of ethinyl estradiol and etonogestrel

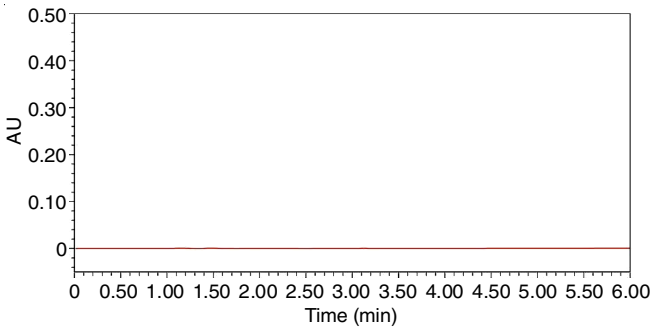


Fig. 3. Placebo chromatogram of ethinyl estradiol and etonogestrel

TABLE-2
RESULTS OF METHOD AND INTERMEDIATE PRECISION

| S. No. | Method precision | | Intermediate precision | |
|---|---|---|---|---|
| | Area of ethinyl estradiol | Area of etonogestrel | Area of ethinyl estradiol | Area of etonogestrel |
| 1 | 406259 | 3256478 | 407896 | 3214154 |
| 2 | 404987 | 3214562 | 403063 | 3225987 |
| 3 | 408502 | 3232588 | 409674 | 3231647 |
| 4 | 402471 | 3275964 | 404897 | 3231496 |
| 5 | 405985 | 3246124 | 401876 | 3267487 |
| 6 | 409374 | 3296983 | 405462 | 3256201 |
| Mean | 100.4 | 99.8 | 100.2 | 99.4 |
| Std. dev. | 0.6 | 0.927 | 0.713 | 0.616 |
| % RSD | 0.6 | 0.93 | 0.71 | 0.62 |

**Linearity:** Different concentrations were analyzed to measure linearity. For each component, the correlation coefficient was higher than 0.999. Table-3 included the slope and y-intercept data, which supported linearity between the concentration and peak regions.

TABLE-3
LINEARITY RESULTS OF ETHINYL ESTRADIOL, ETONOGESTREL

| Parameter | Ethinyl estradiol | Etonogestrel |
|---|---|---|
| Linearity (ppm) | 0.04-0.25 | 3-18 |
| Regression equation | y = 443300.01x + 30330.08 | y = 77745.76x + 56896.75 |
| Slope | 443300.01 | 77745.76 |
| Intercept | 30330.08 | 56896.75 |
| Correlation coefficient ($R^2$) | 0.99929 | 0.99916 |

**Accuracy:** For ethinyl estradiol and etonogestrel, the % recovery lies within the range of 98-102% (Table-4). As a result, the suggested approach is found to be accurate.

TABLE-4
RECOVERY RESULTS OF ETHINYL ESTRADIOL AND ETONOGESTREL

| % Level recovery | Ethinyl estradiol | | | | Etonogestrel | | | |
|---|---|---|---|---|---|---|---|---|
| | H1 | H2 | H3 | H4 | H1 | H2 | H3 | H4 |
| 50 | 0.0075 | 0.00756 | 100.8 | 100.23 | 0.06 | 0.0596 | 99.3 | 100.1 |
| | 0.0075 | 0.00744 | 99.2 | 100.23 | 0.06 | 0.0598 | 99.7 | 100.1 |
| | 0.0075 | 0.00755 | 100.7 | 100.23 | 0.06 | 0.0608 | 101.3 | 100.1 |
| 100 | 0.0150 | 0.01502 | 100.1 | 100.50 | 0.12 | 0.1191 | 99.3 | 99.8 |
| | 0.0150 | 0.01520 | 101.3 | 100.50 | 0.12 | 0.1198 | 99.8 | 99.8 |
| | 0.0150 | 0.01502 | 100.1 | 100.50 | 0.12 | 0.1205 | 100.4 | 99.8 |
| 150 | 0.0225 | 0.02268 | 100.8 | 100.80 | 0.18 | 0.1809 | 100.5 | 100.2 |
| | 0.0225 | 0.02266 | 100.7 | 100.80 | 0.18 | 0.1801 | 100.1 | 100.2 |
| | 0.0225 | 0.02271 | 100.9 | 100.80 | 0.18 | 0.1804 | 100.2 | 100.2 |

H1 = Spiking amount; H2 = Recovered amount; H3 = % recovery; H4 = Mean % recovery

TABLE-5
OUTCOMES OF ROBUSTNESS

| Drug name | Flow (+) (1.1 mL/min) %RSD | Flow (-) (0.9 mL/min) %RSD | Mobile phase (+) (66:34) %RSD | Mobile phase (-) (54:46) %RSD |
|---|---|---|---|---|
| Ethinyl estradiol | 0.55 | 0.75 | 0.31 | 0.67 |
| Etonogestrel | 0.8 | 1.26 | 0.35 | 0.65 |

RSD = Relative standard deviation

**Sensitivity:** The results for the detection limit and quantitation limit were reported as 0.045 and 0.036 ppm, and 0.15 and 1.2 ppm for ethinyl estradiol and etonogestrel, respectively. These values indicate that the method employed exhibited a high level of sensitivity.

**Robustness:** Intentional adjustments had no affect on system suitability parameters like tailing factor or the theoretical plates, RSD, resolution of ethinyl estradiol, etonogestrel. Table-5 displays the results and the variables that affect the suitability of the system. Therefore, it was found that the method could withstand shifts in the conditions under which it was being used.

**Degradation studies:** According to ICH recommendations, different types of forced degradation conditions have been undertaken. During the study, a few degradation products were identified and the results are shown in Table-6.

TABLE-6
RESULTS OF FORCED DEGRADATION PRODUCTS OF ETHINYL ESTRADIOL AND ETONOGESTREL

| Degradation condition | Ethinyl estradiol | | Etonogestrel | |
|---|---|---|---|---|
| | Recovery (%) | Degradation (%) | Recovery (%) | Degradation (%) |
| Control | 100 | 0 | 99.6 | 0.4 |
| Acid | 85.8 | 14.2 | 84.3 | 15.7 |
| Alkali | 87.8 | 12.2 | 87.0 | 13.0 |
| Peroxide | 84.1 | 15.9 | 83.9 | 16.1 |
| Reduction | 91.0 | 9.0 | 88.7 | 11.3 |
| Photo | 97.5 | 2.5 | 99.3 | 0.7 |

**Ethinyl estradiol forced degradation studies:** DP1-DP1 fragmentation mechanism is illustrated in Fig. 4a, the spectrum of ESI reported that the highest extreme [M+H] ion of *m/z* 290.14, which is identified by acid degradation circumstances. The DP1 product, spectrum of MS, reported number of product ions with *m/z* 194.08 (lack of $C_6H_{12}O$) and *m/z* 112.008 (lack of $C_6H_{14}$). For DP2 product, the ESI spectra exhibited the most intense [M+H] ion of *m/z* 294.14 as identified in alkali degradation conditions (Fig. 4b). Number of product ions *viz*. *m/z* 198.10 (lack of $C_6H_{12}O$) and *m/z* 116.02 (lack of $C_6H_{14}$) were also identified in MS spectra of DP2 product. In case of DP3 product, the highest strong [M+H] ion of *m/z* 274.19 was observed due to the peroxide degradation conditions and the other degradation product ions were observed at *m/z* 178.13 (lack of $C_6H_{12}O$) and *m/z* 96.05 (lack of $C_6H_{14}$) (Fig. 4c). For DP4 degradation product, the highest extreme [M+H] ion of *m/z* 290.1437 was detected through the reduction degradation conditions (Fig. 4d). The other product ion under this condition was observed at *m/z* 262.06 (lack of $C_6H_{12}O$) and *m/z* 179.98 (lack of $C_6H_{14}$).

**Etonogestrel forced degradation studies:** The ESI spectrum of DP5 product exhibited the highest strong [M+H] ion of *m/z* 336.18, which is identified under the acid degradation conditions. The number of product ions were also observed at *m/z* 226.11 (lack of $C_7H_{14}O$), *m/z* 132.03 (lack of $C_7H_{14}$) (Fig. 5a). Similarly, in case of DP6 product, the highest [M+H] ion of *m/z* 324.20 was observed in alkali degradation condition (Fig. 5b), and the other product ions at *m/z* 214.13 (lack of $C_7H_{14}O$) and *m/z* 120.05 (lack of $C_6H_{14}$) were found in the MS spectra. Under peroxide conditions, DP7 degradation product displayed the highest strong [M+H] ion of *m/z* 316.20 observed (Fig. 5c) along with few product ions at *m/z* 206.13 (lack of $C_7H_{14}O$) and *m/z* 112.05 (lack of $C_7H_{14}$). Under reduction condition, DP8 degradation product exhibited the highest [M+H] ion of *m/z* -404.16 (Fig. 5d) and the other product ions at *m/z* 294.04 (lack of $C_7H_{14}O$) and *m/z* 200.01 (lack of $C_7H_{14}$) were found in the MS spectra.

## Conclusion

A proposed LC-MS method was recommended for the simultaneous analysis of ethinyl estradiol and etonogestrel in both pure and commercial dose forms, with high accuracy

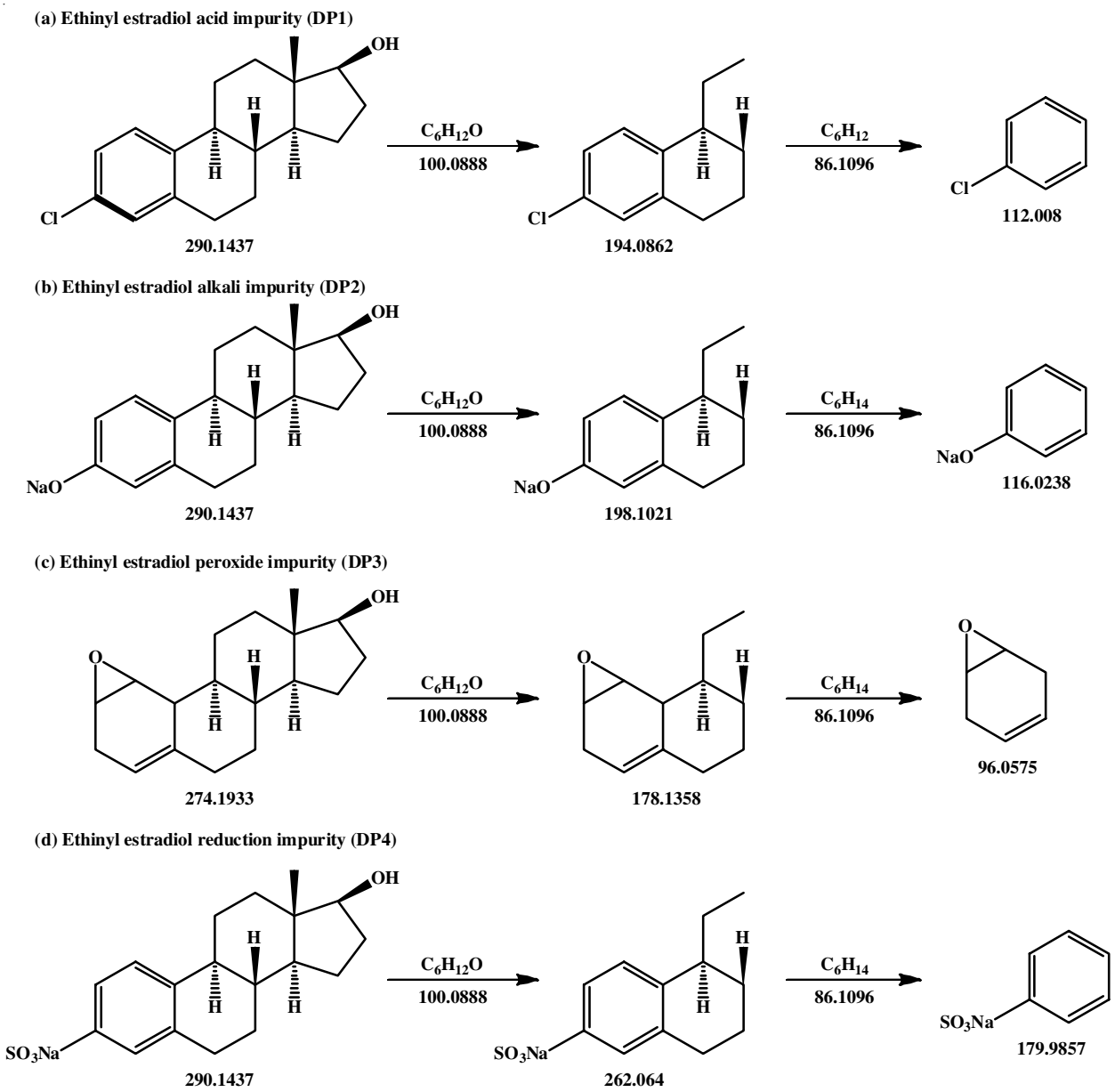


Fig. 4. Mass spectral fragmentation pattern of forced degradation products of ethinyl estradiol under different conditions

and efficiency. The suggested method was extremely precise, accurate and also sensitive. In forced degradation conditions, less degeneration products were formed. For the studies of forced degradation, the plate count, tailing factor, percentage relative standard deviation and degraded percentage are within the limits. This indicates that the process was accurate and reliable. As a result, this method will be used to quantify and identify ethinyl estradiol and etonogestrel in the quality control department.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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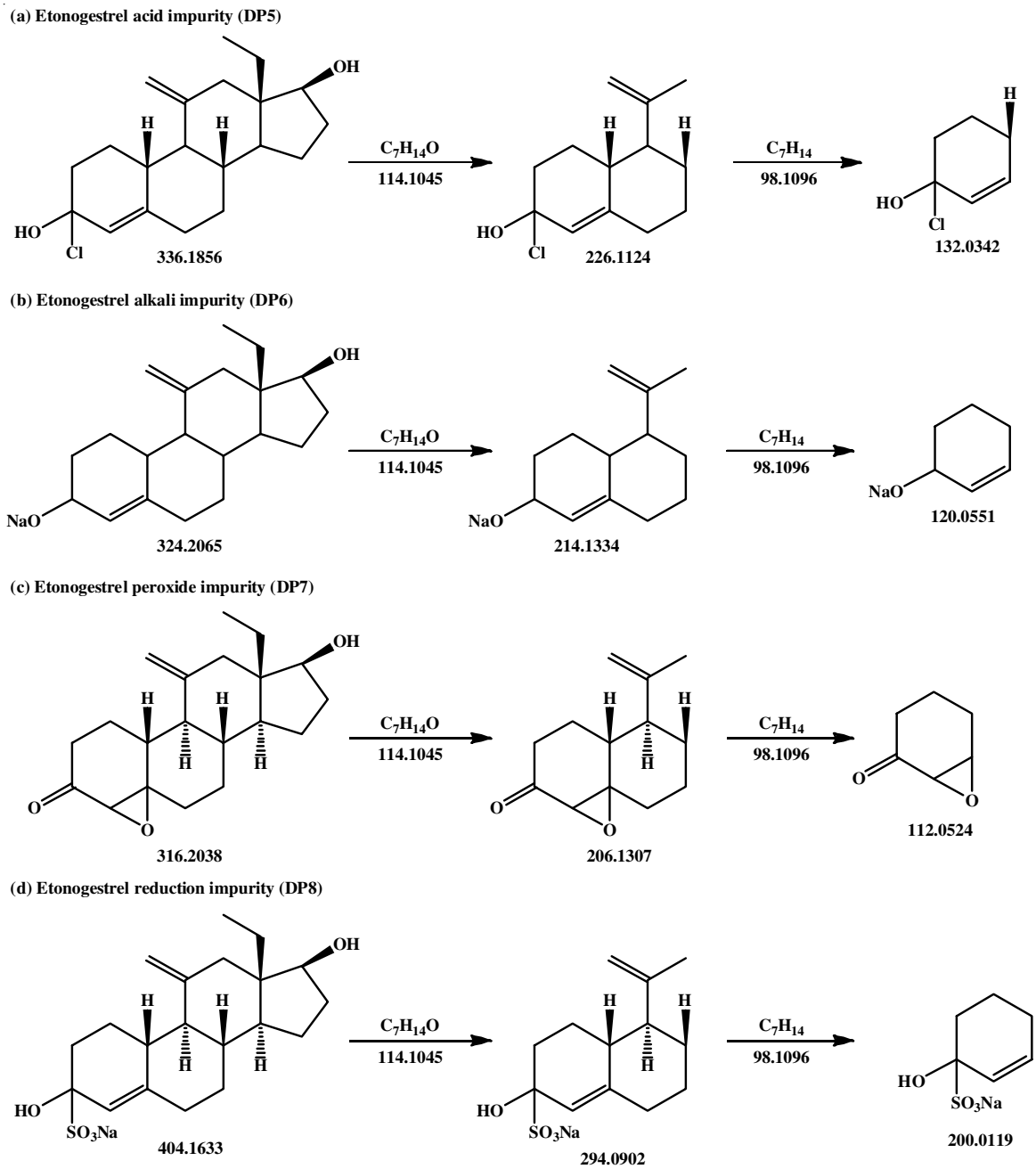


Fig. 5. Mass spectral fragmentation pattern of forced degradation products of etonogestrel under different conditions


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