Analysis of ethylene glycol in animal feed using liquid chromatography electrospray ionization tandem mass spectrometry

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Abstract

A determination method of ethylene glycol in animal feed by liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) was developed. The analysis was performed with a C18 column (150 mm × 4.6 mm, 3.5 µm), and a gradient mobile phase of 0.1 % formic acid solution and acetonitrile. Ethylene glycol and surrogate standard (propylene glycol) were extracted by water at room temperature, derivatized with benzoyl chloride in alkaline medium, and analyzed by LC-ESI-MS/MS. The method has high specificity and selectivity with a detection limit of 0.1 mg/kg, linearity range of 1 - 20 µg/mL, repeatability of 3.08 - 4.41 %, and recovery of 91.1 - 102.0 %, which meet AOAC requirements. This method has been applied to analyze some marketed animal feed samples.

Keywords: *ethylene glycol, animal feed, LC-ESI-MS/MS.*

1. INTRODUCTION

In July 2021, National Institute for Food Control received a feed poisoning case that conducted the death of dogs. The suspected cause of death was acute kidney injury which led to kidney failure. The most common substances that could cause acute kidney injury in dogs are ethylene glycol (EG), medications like non-steroidal anti-inflammatory drugs. Since the dogs did not use medications, so EG had been seen as the center of the case.

Ethylene glycol (EG) (ethane-1,2-diol) is a colorless, syrupy liquid, with molecular mass 62 Da, freely soluble in water, used as an antifreeze for some manufacturing [1]. Upon ingestion, EG is oxidized to a toxic substance, oxalic acid. In cases, sufficient amounts are fatal if the patients are untreated [2]. Several deaths are recorded annually in the United State [3]. In Vietnam, these cases related to EG have not been reported yet.

There are some analytical methods to determine EG with gas and liquid chromatography. In gas chromatography coupled with flame ionization detector, EG could be analyzed by direct injection [4], but the risk is the misinterpretation of interfering peaks. In liquid chromatography coupled with tandem mass spectrometry, direct analysis has the problem because of the small molecular mass, which leads to the low signal and high detection limit [5], indirect analysis with benzoyl chloride has advanced [6]. According to the Schotten-Baumann reaction, the benzoyl derivatization of ethylene glycol was formed in an alkaline aqueous phase with the presence of an excess of benzoyl chloride (Figure 1). A proton and a chloride ion are formed during the reaction, so addition of a strong base was required to neutralize this acidic proton to drive the equilibrium towards the formation of dibenzoyl glycol derivatives.

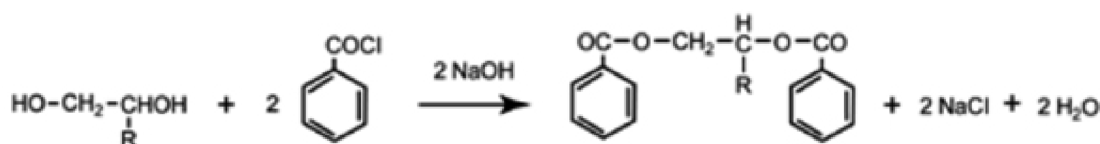

Figure 1. *Dibenzoyl derivatization of glycols [6]*
(R = H: ethylene glycol, R = CH3 : propylene glycol)

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The LC-MS method combined with benzoyl derivatives is known to be the most sensitive method at present, but previous studies have only been performed on plasma, water, soil, cosmetics, and rarely on animal feeds [4-6]. Therefore, this study focused on optimizing an analytical procedure for EG in animal feed samples.

2. MATERIALS AND METHOD

2.1. Chemicals and materials

Reference standards of ethylene glycol (EG) (> 90 %) and surrogate substance propylene glycol (PG) (> 90 %) were obtained from the Laboratory of the Government Chemist (United Kingdom). Acetonitrile, formic acid, n-hexane, and sodium hydroxide were purchased from Merck (Germany). Benzoyl chloride was purchased from Sigma-Aldrich (United States).

The sample used for method validation was the animal feed determined to be free of ethylene glycol. Samples for method application (n = 20) included three animal feed types: dry ready-to-feed, wet ready-to-feed, ready-to-feed paste. An amount of around 100 g of each sample was bought from different stores and markets in Hanoi from July to August 2021, include brands from United States, France, Thailand and non-branded products with no information.

2.2. Equipment

The analysis was performed on LC-MS/MS system with UPLC and triple quadrupole mass spectrometer 6,500 with electron ionization source (ESI) from SCIEX (United States) and C18 Symmetry column (4.6 × 150 mm, 3.5 µm) from Waters (United States). Other types of equipment used in the experiments included analytical balance (Metter Toledo, Switzerland), centrifuge (Sartorius, Germany), vortex mixer (IKA, China), shaker (GFL, Germany), and nitrogen evaporation system.

2.3. Experiments

The method was optimized for the derivatization process and validated on dry ready-to-feed sample. Validation tests were conducted according to AOAC guidelines including selectivity, linearity, precision, recovery, the limit of detection (LOD), limit of quantitation (LOQ), and measurement uncertainty [7].

2.3.1. Standard preparation

(a). Ethylene glycol/ Propylene glycol stock solution 1,000 µg/mL: Weigh accurately about 0.1 g standard to a 100 mL volumetric flask, add distilled water to the mark, shake regularly. Storage at temperature 2 - 8°C, use for three months.

(b). Intermediate ethylene glycol solution 100 µg/mL: Take accurately 1 mL stock solution to a 10 mL volume flask, add distilled water to the mark, shake regularly. Storage at temperature 2 - 8°C, use for three months.

(c). Intermediate propylene glycol solution 10 µg/mL: Take accurately 1mL stock solution to 100 mL volume flask, add distilled water to the mark, shake regularly. Storage at temperature 2 - 8°C, use for three months.

(d). Calibration solution: Use a pipette to take each 10, 20, 50, 100, 200 and 500 µL solution (b) and 1 mL solution (c) to 50 mL centrifuge tube. Add 30 mL distilled water, shake regularly. Add 4 mL Sodium hydroxide and 400 µL benzoyl chloride, shake regularly for ten minutes and hold on for ten minutes. Add 10 mL hexane, shake for 20 minutes. The hexane layer was moved to the other 15 mL centrifuge tube and taken to dryness with a gentle nitrogen stream. The final extract was made up to 1 mL by acetonitrile, filtered through 0.2 µm membrane to a vial, filtered through 0.2 µm membrane to the vial, and analyzed by LC-MS/MS. The calibration curve was accepted with at least three points.

2.3.2. Sample preparation

For the sample preparation, we have referred the research of The National Institute for Occupational Safety and Health [1] and also investigated the derivatization conditions. At the time of collection (purchase), the feed was grounded (or minced by an electric mincer), and homogenized thoroughly.

Weigh accurately about 10.0 g homogenized sample to 50 mL centrifuge tube. Add 1 mL solution (c) to sample. Add 15 mL hexane, shake horizontally for 15 minutes. Centrifuge at the speed of 6,000 rpm for five minutes. Use a Pasteur pipette to remove the hexane layer. Add 15 mL distilled water and shake regularly. Decant the supernatant to another 50 mL centrifuge tube. Repeat extraction with other 15 mL of distilled water. Combine and shake well. Add 4 mL of 4 M sodium hydroxide solution and 400 µL benzoyl chloride, shake regularly for ten minutes, and let still for ten minutes. Benzoyl derivatives were extracted by adding 10 mL hexane, shaking for 20 minutes. The hexane layer was moved to the other 15 mL centrifuge tube and taken to dryness with a gentle nitrogen stream. The final extract was made up to 1 mL by ACN, filter through 0.2 µm membrane to a vial.

2.3.3. LC-MS/MS conditions

A Symmetry C_{18} column (4.6 × 150 mm, 3.5 µm) was used. The mobile phase system consisted of solution A (0.1 % formic acid aqueous solution) and B (ACN). A gradient program started with 10 % B to 1.0 minutes, increase to 80 % B in the next four minutes, then decrease 10 % in the next 0.5 minutes and hold for 2.5 minutes till the end. The flow rate was set at 0.5 mL/min.

The triple quadrupole mass spectrometer 6,500 was equipped with an electron ionization source (ESI) source with the following parameters: ESI temperature of 400°C, the capillary temperature of 350 °C, spray voltage of 4.5 kV, sheath gas flow of 35 arbitrary units, the auxiliary gas flow of 32 arbitrary units. The mass spectrometer was operated in the multiple reaction monitoring (MRM). For data collection and analysis, the quantitation was conducted by Analyst software (Sciex). Mass spectrometry conditions and mass fragments are shown in Table 1.

Table 1. *Mass spectrometry conditions and mass fragments of analyte and the surrogate*

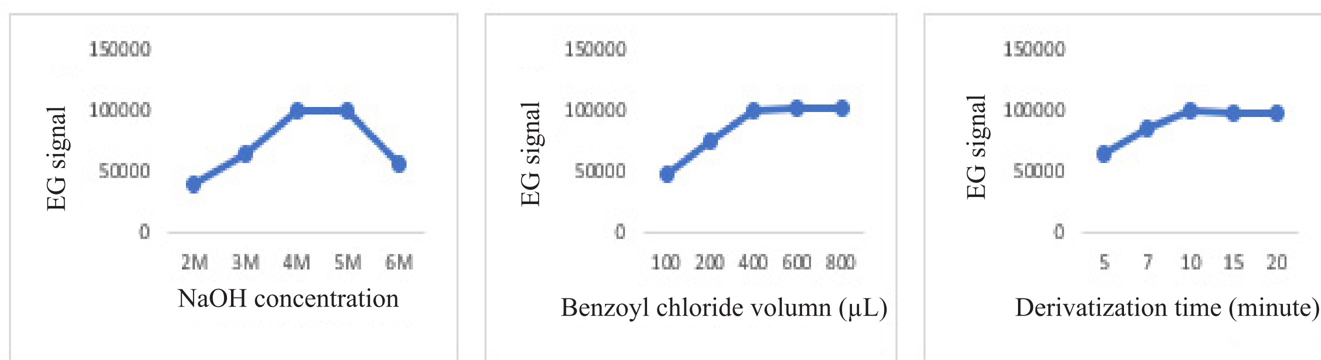
Compounds	***Precursor ion (m/z)***	***Product ions (m/z)***	***Collusion Energy (V)***
EG	271.0	149.2*	19
		105.2	41
PG	285.0	163.0*	10
		105.0	20

**: Quantitative ion*

3. RESULTS AND DISCUSSION

3.1. Derivatization conditions

The amounts of benzoyl chloride and sodium hydroxide concentration used, as well as the reaction time of the experimental, were optimized to maximize the amount of dibenzoyl glycol derivatives (Figure2).



a. Amounts of sodium hydroxide *b. Benzoyl chloride volume* *c. Reaction time*

Figure 2. *Derivatization process optimizing*

The results of the derivatization optimization process showed that using 4 M sodium hydroxide solution and 400 µL of benzoyl chloride in ten minutes, the dibenzoyl derivatization rate of ethylene glycol formed would be the highest. This result has no significant deviation from previous studies [6].

3.2. Method validation

3.2.1. Specificity

The result showed that both the analyte and the internal standard have an identification point (IP) score of four which is satisfactory for analysis on mass spectrometry.

The blank, standard solution and spiked-blank samples with concentrations of EG at 0.2 mg/kg were analyzed using the optimized method. The chromatograms showed that the blank sample shows no signal at the retention time of the analyte. The spiked-blank sample has a signal at a retention time of 3.63 minutes that coincides with the retention time of the standard solution (3.62 minutes), with a difference of not more than 5 %.

For mass spectrometry, the ratio of ions is the criterion to confirm the presence of an analyte. The ion ratio is the percentage of the lower signal ions divided by the higher signal ions of the same precursor ion. To calculate ion ratio, standard solution and spiked-blank samples were analyzed, then compare the obtained ion ratio. The result showed that the ion ratio of the standard solution and the spiked sample is 8.12 % and 5.57 %, respectively. The deviation of the spiked sample when compared with results of standard solution varied 4.06 - 12.18 %, in the range of ± 50 %, conformed European regulations (EC/657/2002) [8]. Therefore, the method had high specificity, suitable for EG analysis.

Table 2. *Ion ratio of Ethylene glycol analyzed by LC-MS/MS*

Analyte	*Ion ratio in standard solution*	*Permitted tolerances*	*Maximum permitted tolerances*	*Ion ratio in spike sample*
EG	8.12 %	± 50 %	4.06 % - 12.18 %	5.57 %

3.2.2. Linearity

Spiking standard solution into the blank and solvent at concentrations of 1 - 20 mg/L, do the same with the sample and analyzed by LC-MS/MS method to determine the linearity of the method. The standard curve representing the dependence between Speak of standard/Speak of internal standard and the corresponding concentration was made by the instrument's software. The calibration curve was presented in Figure 3.

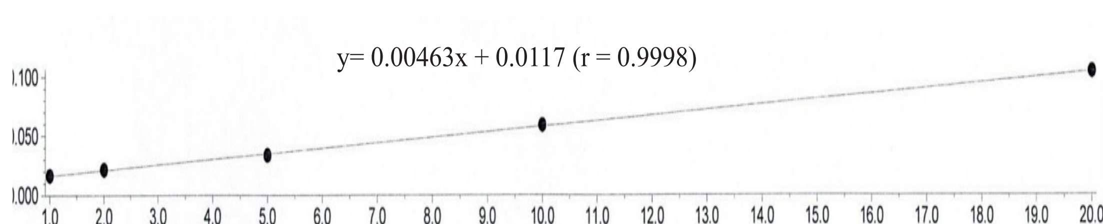


Figure 3. *Ethylene glycol calibration curve*

The result showed good linearity with the variance coefficient being higher than 0.99 with a bias of < 15 % for all values. The slope of the calibration curve in matrix and solvent was 0.157 and 0.00463, respectively.

3.2.3. Limit of detection - Limit of quantification

The analysis of EG was repeated six times to determine the S/N ratio. The of detection is the concentration at which S/N $\geq$ 3. The limit of quantification is the limit at which S/N = 10 or LOQ = 3.3 × LOD (Figure 4) .

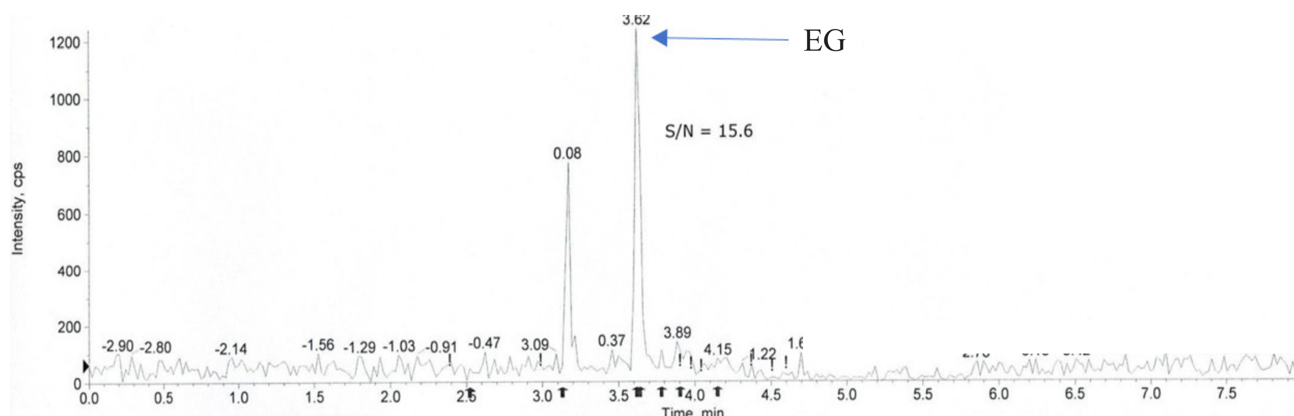


Figure 4. *Ethylene glycol at detection level 0.1 mg/kg*

3.2.4. Precision and recovery

Precision and recovery are two important factors in evaluating the effectiveness of an analytical method. We performed analysis of standard addition on the real sample to conduct the simultaneous determination of these two factors, and evaluate the repeatability and recovery of EG at three concentration levels 0.1, 0.2, and 0.5 mg/kg, n = 6. For reproductivity evaluation, two staff performed the same analysis on the same concentration 0.2 mg/kg, six times for each.The results of the recovery and precision calculations were presented in Table 3.

Table 3. *Precision and recovery*

Parameters	***Values***
Repeatability (RSDr)	3.08 - 4.41 %
Reproductivity (RSDR)	4.21 %
Recovery range	91.1 - 102.0 %

3.2.5. Uncertainty

The measurement uncertainty (U) was evaluated based on recovery and reproducibility according to “Guidelines on Estimation of Uncertainty of Results” (CAC/GL 59-2006) [9] and ISO 21748:2004 “Guidance for the Use of repeatability, reproductivity and trueness estimates in measurement uncertainty estimation” [10]. The result showed that measurement uncertainty of the method was 13.3 %.

3.3. Application for real samples

The procedure, after being optimized and validated, was applied for the analysis of 20 animal feed samples, each sample was prepared in duplicate. Animal feeds were collected from a variety of brands and non-brands in Hanoi, such as supermarkets, local stores, vet clinics, and pet stores. The supermarket and vet clinic only sold the brands. However, the local and pet store not only had the brands, but also the non-brands. In Figure 5, number of samples purchased from each source was depicted. While supermarkets and vet clinics only sole brand products, local stores and pet stores sole both brand and non-brand feeds.

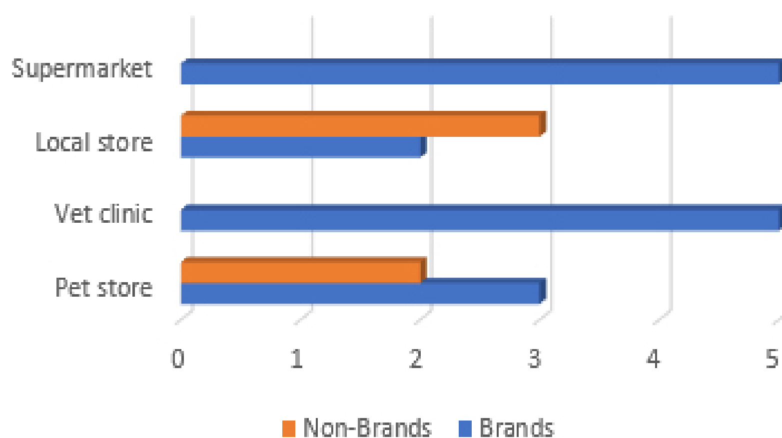


Figure 5. *The total number of investigated samples for each type of brand and type of location*

Each sample was homogenized before analysis. Among 20 samples, five samples which detected with EG at different concentrations of 3.0, 3.5, 5.2, 8.9, and 10.0 mg/kg, were all non-brands. It can be concluded that brand animal feeds are well controlled for safety, and products without labels are not. According to United States Agency for Toxic Substances and Disease Registry (ATSDR), the chronic oral Minimal Risk Levels (MRLs) for EG is 0.8 mg/kg/day has been derived for acute-duration oral exposure (≤ 14 days) [11]. However, based on the same endpoint as Environmental Protection Agency's reference dose, the MRL is 2.0 mg/kg/day [12]. It means that, the MRL is still not unified globally. On the other hand, although there are many potential risks to animal health, up to now, Vietnam has not had regulations related to the allowable levels of EG in animal feed. The method has been designated as a method for state management in the field of animal feed safety. The results of the study contribute to providing data for Vietnamese regulatory agencies

3.4. Limitation

The method uses PG as a surrogate, in both sample processing and analysis simultaneously in the instrument. This could not be as accurate as using the isotope internal standard of EG but can be used alternatively instead.

4. CONCLUSION

So far, a sensitive method for analysis of EG in animal feed has been reported. The method was validated and met the AOAC International requirements for selectivity, specificity with a good linear range of 1 ÷ 20 mg/kg. LOD and LOQ were 0.03 and 0.1 mg/kg. The recoveries of EG were from 91.1 to 102.0 % and the relative standard deviations were within the range of 3.08 ÷ 4.41 %. The measurement uncertainty of the method for animal feed was 13.3 %. The method is qualified to conduct interlaboratory validation. It can become a standard method that will contributing to safety confirmation of EG-containing products including food and feed on the market.

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Phân tích ethylene glycol trong thức ăn chăn nuôi bằng sắc ký lỏng ion hóa phun điện tử ghép nối khối phổ hai lần

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Tóm tắt

Phương pháp xác định ethylene glycol trong thức ăn chăn nuôi bằng sắc ký lỏng kết hợp với ion hóa phun điện tử ghép nối khối phổ hai lần (LC-ESI-MS/MS) đã được xây dựng và thẩm định. Phương pháp này được thực hiện với cột C18 (150 mm × 4,6 mm, 3,5 µm), pha động gradient bao gồm acid formic 0,1 % và acetonitrile. Ethylene glycol và chất chuẩn đồng hành (propylene glycol) được chiết xuất bằng nước ở nhiệt độ phòng, tạo dẫn xuất bằng benzoyl chloride trong môi trường kiềm và được phân tích bằng LC-ESI-MS/MS. Kết quả thẩm định cho thấy phương pháp này có độ đặc hiệu và độ chọn lọc cao với giới hạn phát hiện 0,1 mg/kg, khoảng tuyến tính 1 - 20 µg/mL, độ lặp lại 3,08 - 4,41 %, và độ thu hồi 91,1 - 102,0 %, đáp ứng các yêu cầu của AOAC. Phương pháp đã được áp dụng để phân tích một số thức ăn chăn nuôi phân phối trên thị trường.

Keywords: *ethylene glycol, animal feed, LC-ESI-MS/MS.*