Determination of ethylene oxide and 2-chloro-ethanol using gas chromatography-tandem mass spectrometry: Internal standard and stainless-steel ball coupled with QuOil in high-fat food extraction

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Abstract

A determination method of ethylene oxide (ETO) and 2-chloro-ethanol (2-CE) in foods, especially those with high-fat content, by gas chromatography-tandem mass spectrometry (GC-MS/MS) was developed. The analysis was performed with a TG-WAX column (60 m × 0.32 mm × 1 µm), with a programmable temperature vaporization-large volume injection method (PTV-LVI). ETO, 2-CE, and isotope internal standard 2-chloro-ethanol-d4 (2-CE-d4) were extracted by the QuOil (a variation of QuEChERS method), in combination with the use of stainless-steel balls, especially effective with high-fat matrices and analyzed by gas chromatography-mass spectrometry (GC-MS/MS). For both compounds, the method has high specificity and selectivity with a detection limit of 0.003 mg/kg, the linear range of 0.01 - 0.2 mg/kg, reproducibility and recovery meet AOAC requirements. This method has been applied to analyze 1668 samples during the period 2021-2022 at the National Institute for Food Control (NIFC).

Keywords: *ethylene oxide, 2-chloro-ethanol, QuOil, stainless steel balls, GC-MS/MS.*

1. INTRODUCTION

In August 2021, The Food Safety Authority of Ireland (FSAI) announced the decision to recall a number of Vietnamese instant noodle products for containing ethylene oxide (ETO) - a substance that was not allowed to be used in foods sold in the European Union (EU). The FSAI stated that consuming products contaminated with ETO does not pose an acute risk, however, long-term consumption could cause health problems [1]. This month,

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another number of Vietnamese instant noodle products was recalled in Norway, according to the product containing 0.052 mg/kg ETO, in violation of EU Council Directive 91/414/EEC [2].

ETO is a colorless, flammable gas, mainly used as an intermediate in the production of ethylene glycol (antifreeze), textiles, detergents, solvents, adhesives, raw materials for the production of polyethylene terephthalate (PET) and other products. A small portion (~0.05% of world production) is also used to disinfect medical instruments and combat mold and bacteria in foods such as spices, nuts, and oilseeds, and some food additives such as guar gum and locust bean gum [3]. ETO is rarely found in foods because of low evaporation temperature (> 10°C) or conversion reaction to 2-chloro-ethanol (2-CE), 2-bromo-ethanol, and ethylene glycol (EG), most prominent of which be 2-CE (Figure 1) [4].

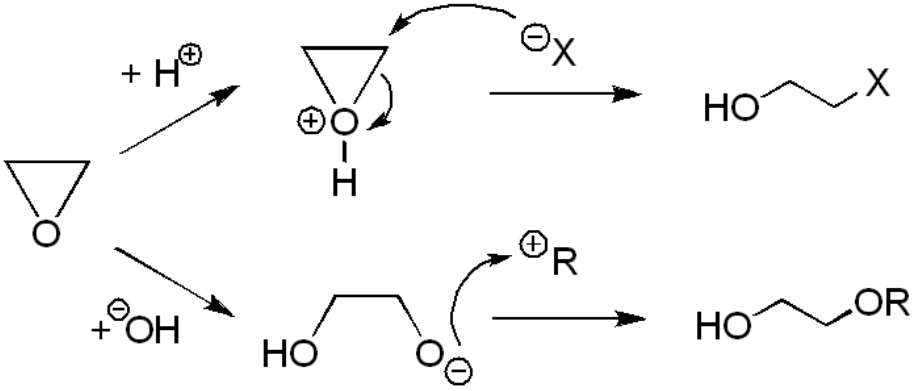

Figure 1. *Conversion diagram of ETO [4]*
Example: 2-CE (X = Cl^-), EG (R = H^+)

ETO is used for sterilization due to the mutagenesis mechanism of DNA damage in microorganisms, thus also potentially toxic, mutagenic and carcinogenic in humans. Several experiments have shown that ETO and its metabolite 2-CE increase the risk of lymphoma and breast cancer. The German Federal Institute for Risk Assessment (BfR) recently evaluated the toxicity of 2-CE. Given the inconclusive toxicity of 2-CE, they decided to take a precautionary approach and considered the toxicity of 2-CE to be comparable to that of EO. Therefore, 2-CE is included in the current ETO maximum residue level (MRL) definition.

Maximum residue level MRL of ETO = ETO + 2-CE (expressed in EO)

In Europe, ETO is classified as a banned pesticide product. ETO is not permitted for food sterilization; ETO is classified in group 1B for mutagenic carcinogenicity and reproductive toxicity, respectively, and class 3 for acute toxicity, according to Reg. 1223/2009/EC of the Council of Europe (Table 1) [5].

Table 1. *ETO and 2-CE maximum limits in some countries*

Location	***Analyte***	***Food matrices***	***MRL (mg/kg)***
European Union [6]	ETO (sum of ETO and 2-CE expressed as ETO)	Thirteen different food groups	0.01 to 0.1
United States [7]	ETO	Herb, spice and dried vegetable	7 - 50
	2-CE		940
Canada [8]	ETO	Dried vegetables and sesame seeds and any processed food product	7
	2-CE		940
Japan [9]	2-CE	Food	0.1
Australia and New Zealand [10]	ETO	Herbs, spices, include dried seasoning vegetables	20

In the Technical Meeting on Ethylene Oxide on 20/01/2022 [11] with Representatives from EU Member States, Norway, Switzerland, the European Food Safety Authority (EFSA), the European Commission (DG Health and Food Safety), and the EU Reference Laboratories (EURLs), the European Commission explains that there have been a number of recent incidents involving the presence of ETO in food products from food additives containing these substances. The difficulty of identifying the source of ETO present in food-be it through illegal sterilization of food additives or any other reason-has made it difficult to enforce the substance in food. To avoid future enforcement challenges and recall incidents, the European Commission has clarified that the presence of ETO, regardless of its origin, is not allowed for all food additives. The commission also set a limit of 0.1 milligrams per kilogram for ETO, including 2-CE expressed as ETO, in some additives treated with the substance. Currently, in Vietnam, there is no regulation on the maximum allowable residue limit of ETO on food.

There are many methods in the world that can be used to analyze ETO and 2-CE in foods, with a variety of sample processing methods with analytical methods using liquid chromatography and gas chromatography, with or not combined with transform derivation (Table 2).

Table 2. *Analytical methods*

Matrices	***Sample preparation***	***Analytical method***	***LOQ (mg/kg)***	***Ref.***
Food	***QuEChERS***	GC-MS/MS	0.01	[12]
Oil	***QuOil***	GC-MS/MS	0.01	[13]
Processed food	***Steam distillation + derivatives***	GC-ECD	0.05	[14]
Spices	***Headspace***	GC-FID	1.00	[15]
Pepper	***Steam distillation***	GC-MS/MS	55.0	[16]

In Vietnam, there is no official method for testing ETO and 2-CE in food. Through reference to a number of studies, the QuEChERS sample processing method combined with direct sample analysis by GC-MS/MS equipment is suitable for the food sample background and has the lowest required sensitivity according to the European MRL (0.01 mg/kg). However, for matrix with high fat content, this sample treatment method has some limitations in sample dispersion. Therefore, this study performed analysis of the total content of ETO and 2-CE converted to ETO by GC-MS/MS method on the basis of the European Reference Laboratory for Pesticides Requiring. Single Residue Methods: QuOil-Method (CEN/TS 17062:2019 modified) - a variation of QuEChERS method [13].

2. MATERIALS AND METHOD

2.1. Chemicals and materials

Reference standards of ETO (1,000 µg/mL in Triacetin) was obtained from the Laboratory of the Government Chemist (United Kingdom). 2-CE and 2-CE-d4 were supplied from HPC Standards GmbH (Germany).

Acetonitrile, magnesium sulfate and sodium chloride were purchased from Merck (Germany). Trisodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$) and sodium hydrogen citrate sesquihydrate ($C_6H_6Na_2O_7.1.5H_2O$) were purchased from Sigma-Aldrich (United States). Primary Secondary Amine (PSA), C18 powder were purchased from Agilent (United States). Water was obtained from Laboratory Water Purification Systems (Merck Millipore).

The sample used for method validation was the high-fat matrices determined to be free of ETO and 2-CE. Samples for method application varied from high-fat types: oil, oily spices, noodles. One thousand, six hundred and sixty-eight (1668) samples listed in this study were collected during the period 2021-2022 at the National Institute for Food Control (NIFC).

2.2. Equipment

The analysis was performed on Thermo TSQ 9000 GC-MS/MS system with a programmable temperature vaporization-large volume injection method (PTV-LVI) and TG-WAX - an acid optimized polyethylene glycol packed column (60 m × 0.32 mm × 1 µm) from Thermo Fisher Scientific (United States). Other types of equipment used in the experiments included analytical balance (Metter Toledo, Switzerland), centrifuge (Sartorius, Germany), vortex mixer (IKA, China) and shaker (GFL, Germany). Stainless-steel balls (I.d. 5 mm) were purchase from ADC Chemical (Vietnam).

2.3. Experiments

The method was optimized for the QuOil and validated on high-fat sample. Validation tests were conducted according to AOAC guidelines including selectivity, linearity, precision, recovery, limit of detection (LOD), limit of quantitation (LOQ), and measurement uncertainty [17].

2.3.1. Standard preparation

(a). Ethylene oxide stock solution 100 µg/mL: Pipette accurately 1 mL standard (1000 µg/mL in Triacetin) to a 10 mL volumetric flask, add acetonitrile to the mark, shake regularly. Storage at temperature - 20°C, use for twelve months.

(b). 2-chloro-ethanol stock solution 1000 µg/mL: Weigh accurately 20 mg standard to a 20 mL volumetric flask, add acetonitrile to the mark, shake regularly. Storage at temperature 2 - 8°C, use for twelve months.

(c). 2-chloro-ethanol intermediate solution 100 µg/mL: Pipette accurately 1 mL stock solution to a 10 mL volumetric flask, add acetonitrile to the mark, shake regularly. Storage at temperature 2 - 8°C, use for three months.

(d). Mix-standard intermediate solution 1 µg/mL: Pipette accurately 1 mL from (a) and (c) to a 100 mL volumetric flask, add acetonitrile to the mark, shake regularly. Storage at temperature - 20°C, use for three months

(e). 2-chloro-ethanol-d4 stock solution 1000 µg/mL: Weigh accurately 10 mg standard to a 10 mL volumetric flask, add acetonitrile to the mark, shake regularly. Storage at temperature 2 - 8°C, use for twenty-four months.

(f). 2-chloro-ethanol-d4 intermediate solution 100 µg/mL: Pipette accurately 1 mL stock solution (e) to a 10 mL volumetric flask, add acetonitrile to the mark, shake regularly. Storage at temperature 2 - 8°C, use for twelve months.

(g). 2-chloro-ethanol-d4 intermediate solution 1 µg/mL: Pipette accurately 100 µL stock solution (e) to a 10 mL volumetric flask, add acetonitrile to the mark, shake regularly. Storage at temperature 2 - 8°C, use for three months.

(h). Calibration solution 10 - 200 ng/mL: Pipette aspirate exactly every 10; 20; 40; 100 and 200 µL (d) and 50 µL (g) were added to the vial, added acetonitrile to 1 mL, to obtain calibration solutions of 10; 20; 40; 100 and 200 ng/mL, with 50 ng/mL of internal standard concentration each. Freshly prepared working standard solution for each analysis.

2.3.2. Sample preparation

At the time of collection (purchase), the food was homogenized thoroughly, at least 200 g each, before further preparation.

Homogenized sample was weighed accurately about 2.0 g to 50 mL centrifuge tube. Then, 500 µL solution (g) was added to sample, follow through with 10 mL of acetonitrile containing 5 %, v/v of water and 10 stainless steel balls as extraction aids. The tube was shaken horizontally for 15 min and centrifuged at the speed of 6000 rpm for 5 min. The liquid layer was dispersed to SPE cleanup with C18/PSA/MgSO4 (25/25/150 mg per milliliter extract solution) to remove lipids and fatty acids. The extract is transfer to vials for GC-MS/MS analysis.

2.3.3. GC-MS/MS conditions [13]

A TG-WAX column (60 m × 0.32 mm × 1 µm) was used. Helium was used as carrier gas, flow-rate 1mL/min. A gradient program started with column initial temperature: 40°C,

keep for 2 min then increase 20°C every min until reaching 150°C, maintain for 1 min; increase 30°C every min to 240°C, keep for 3 min. The PTV-LVI with injection volume 4 µL and split line 5:1; syringe initial temperature: 90°C, hold for 0.8 min, increase 12°C/sec to 250°C, hold for 10 min.

The mass spectrometer was operated in the multiple reaction monitoring (MRM) with electron ionization (EI) source 70eV. The transfer line temperature was set at 250°C and ion source temperature at 230°C. For data collection and analysis, the quantitation was conducted by Excalibur (Thermo Scientific). Mass spectrometry conditions and mass fragments are shown in Table 3.

Table 3. *Mass spectrometry conditions and mass fragments of analyte and the surrogate*

Compounds	***Precursor ion (m/z)***	***Product ions (m/z)***	***Collusion Energy (eV)***
EO	44	14.1*	20
		29.1	5
2CE	80*	31.1*	5
	82	31.1	5
2CE-d4	84*	33.1*	5
	86	33.1	5

*Note: *: Quantitative ion*

3. RESULTS AND DISCUSSION

3.1. QuOil sample preparation with extraction aids

Although the QuEChERS method is widely used to process samples of ETO and its metabolites, it also has limitations for samples with high fat content. ETO and its metabolites may not be fully extracted. The extraction of 2-CE was found to be considerably delayed compared to other analytes. It is thus of high importance to use extraction aids (e. g. stainless steel balls) during extraction to disintegrate the sample and improve the accessibility of the residues [18].

Comparative experiments between QuEChERS and QuOil, with or without stainless steel balls, at initial extraction times of 15, 30 and 45 min, were performed on an oil seasoning sample matrix, in triplicate for each. The results were shown in Figures 2 and Figure 3.

The results showed that extraction by the QuOil method (without extraction salt), incorporating steel balls, both increased the ability to separate the analyte from the sample matrix, and at the same time reduced the extraction time to 15 minutes. This is consistent with previous research on sesame seed samples [18]. The QuOil method with extraction aids presents a performance advantage when applied to the analysis of high-fat samples.

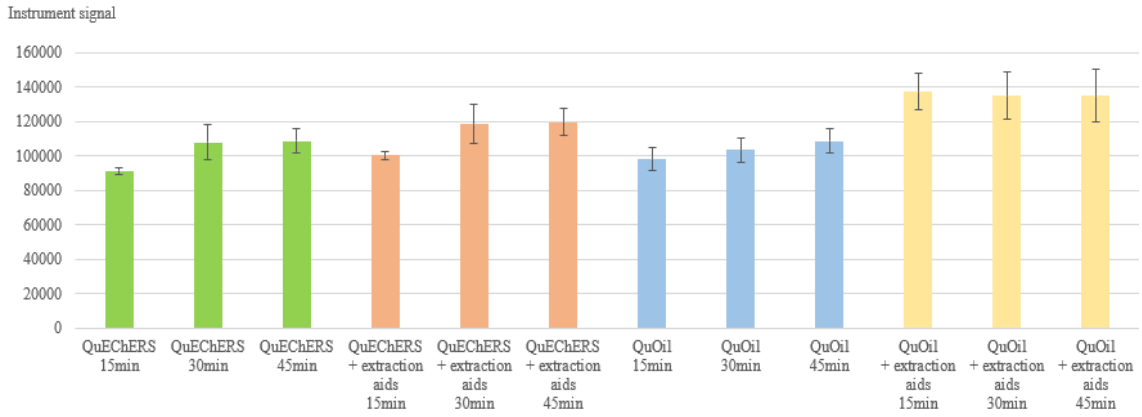

Figure 2. *Sample preparation with and without extraction aids on oil seasoning sample*

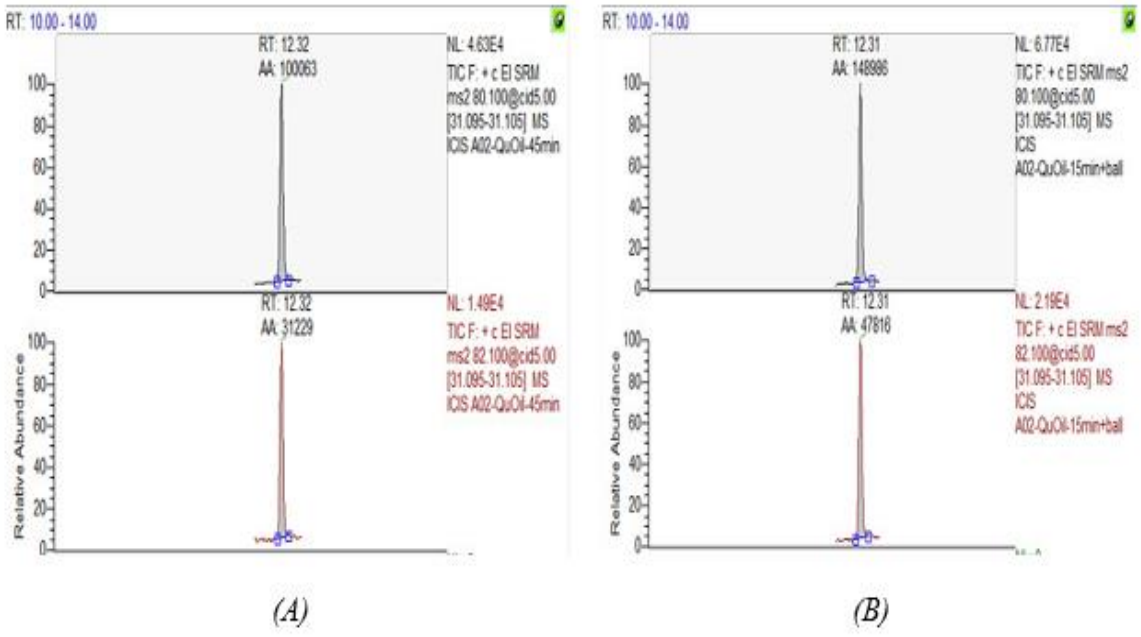

Figure 3. *Chromatograms of 2-CE by QuOil 45min (A) and QuOil with extraction aids 15 min (B) in oil seasoning sample*

3.2. External standard versus internal standard

Different matrices have different effects on the analytical results. The effect of the matrix was performed by standard spiking at levels on an oil seasoning blank. The results were shown in Figure 4.

The standard curve had a correlation coefficient less than 0.99, the deviation of the points did not meet the requirements of AOAC. To overcome this limitation, an internal standard was used. An internal standard in analytical chemistry is a chemical substance that is added in a constant amount to samples, the blank and calibration standards in a chemical analysis. It is used to correct for the loss of analyte during sample preparation, injection and ionization. In the framework of this experiment, the internal standard used was 2-CE-d4,

same as previously studies [18-19]. This internal standard was intended for general use for the simultaneous analysis of ETO and 2-CE, because ethylene oxide-d4 (ETO-d4), internal standard for ETO, was out of stock during the preparation and conduct of this experiment. Calibration curves with integrated internal standards are more suitable for analysis (Figure 5).

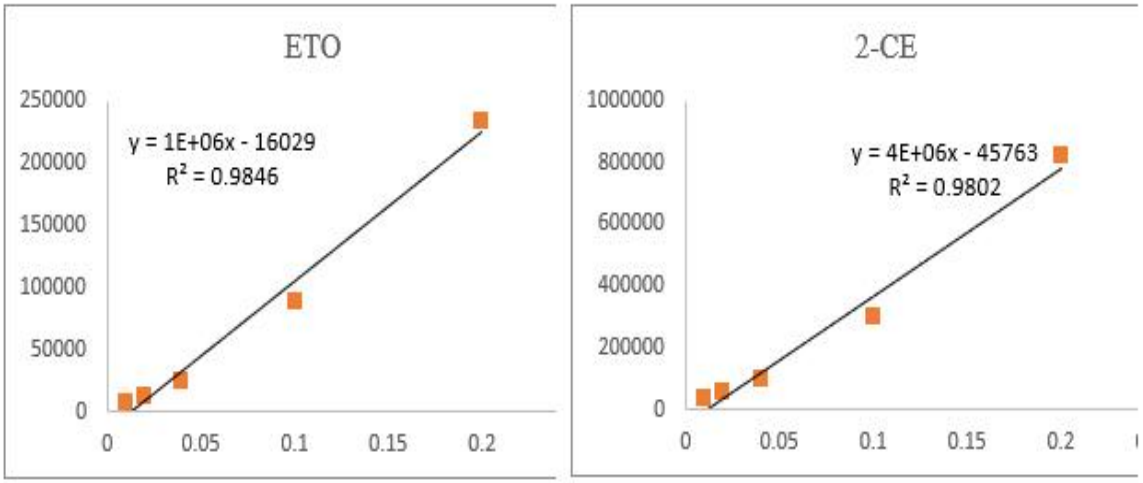

Figure 4. *ETO and 2-CE calibration curves, without internal standard*

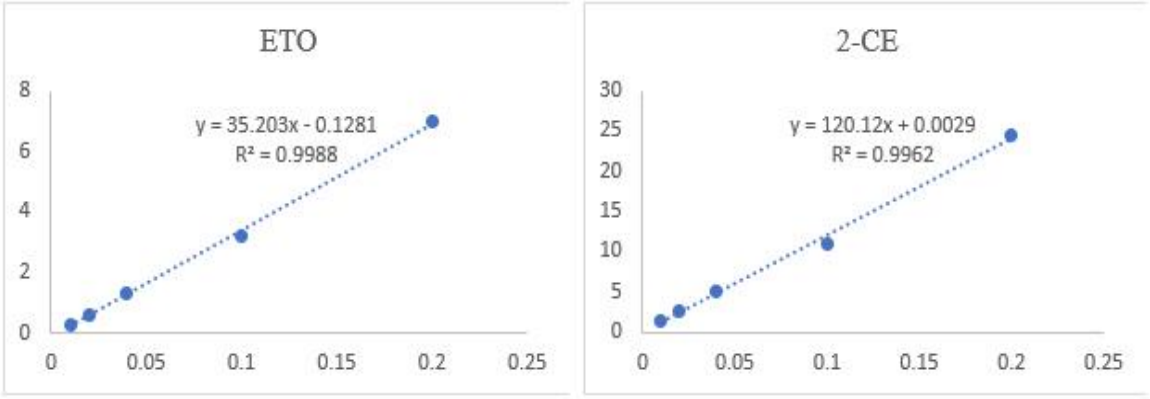

Figure 5. *ETO and 2-CE calibration curves*

3.3. Method validation

3.3.1. Specificity

The result showed that both the analyte and the internal standard have an identification point (IP) score of which is satisfactory for analysis on mass spectrometry according to EU 2021/808 [20].

The blank, standard solution and spiked-blank samples with concentrations of ETO and 2-CE at 0.01 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 signals at retention times that coincides with the retention times of the standard solution (ETO: 5.87 min; 2-CE: 12.3; 2-CE-d4: 12.3), with a difference of not more than 2 %.

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, 0.01 mg/L standard solution and 0.01 mg/kg spiked-blank samples were analyzed in triplicate, then compare the obtained ion ratio. The result showed that the ion ratio of the standard solution and the spiked sample is 4.74 % and 4.63 % for ETO, 31.42 % and 32.45 % for 2-CE, respectively. The deviation of the spiked sample when compared with results of standard solution conformed European regulations (EU 2021/808) (Table 4) [20]. Therefore, the method has high specificity, suitable for ETO and 2-CE analysis.

Table 4. *Ion ratio of ETO and 2-CE by GC-MS/MS*

Analyte	*Ion ratio in standard solution*	*Permitted tolerances (EU 2021/808)*	*Maximum permitted tolerances*	*Ion ratio in spike sample*
ETO	4.74 ± 0.24%	± 50%	2.37% - 7.11%	4.63 ± 0.21%
2-CE	31.42 ± 1.62%	± 15%	26.7% - 36.1%	32.45 ±1.85%

3.3.2. Linearity

Spiking standard solution into the blank and solvent at concentrations of 0.01 - 0.2 mg/kg, do the same with the sample and analyzed by GC-MS/MS method to determine the linearity of the method. The standard curve representing the dependence between $S_{peak\ of\ standard}/S_{peak\ of\ internal\ standard}$ and the corresponding concentration was made by the instrument's software. The calibration curve, presented in Figure 5, showed good linearity with the variance coefficient being higher than 0.99 with a bias less than 15% for all values.

3.3.3. Limit of detection - Limit of quantification

The analysis was repeated 6 times to determine the S/N ratio. The limit of detection is the concentration at which S/N ≥ 3. The limit of quantification is the limit at which S/N = 10 or LOQ = 3.3 × LOD.

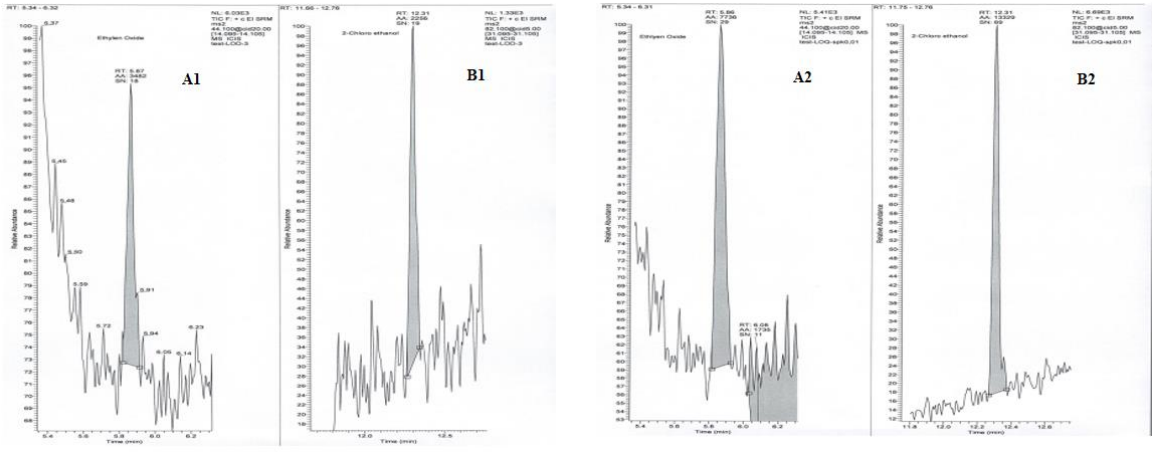

Figure 6. *ETO (A) and 2-CE (B) at detection (1) and quantification (2) level*

According to the survey results, the limit of quantification (LOQ) of ETO and 2-CE at the concentration of 0.01 mg/kg with the average value S/N is 29 and 69, respectively. The laboratory recommended limit of quantification (LOQ) of this method was 0.01 mg/kg, the corresponding limit of detection (LOD) was 0.003 mg/kg. The chromatogram of ETO at the LOQ level was presented in Figure 6.

3.3.4. 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 ETO and 2-CE at 3 concentrations levels 0.01, 0.02, and 0.04 mg/kg, n = 6. For reproducibility evaluation, two staff performed the same analysis on the same concentration 0.01 mg/kg, 6 times for each. The results of the recovery and precision calculations were presented in Table 5.

***Table 5**. Precision and recovery*

Parameters	***ETO***	***2-CE***
Repeatability (RSD_r)	1.84 - 6.36 %	0.75 - 3.13 %
Reproducibility (RSD_R)	3.26 %	1.80 %
Recovery range	94.0 - 110.0 %	99.6 - 107.6 %

3.3.5. 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) [21] and ISO 21748:2004 "Guidance for the Use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation" [22], with the following formula:

$$U = 2u$$

$$u = \sqrt{(u_{Rw})^2 + (u_{bias})^2}$$

$$u_{bias} = \sqrt{(RMS_{bias})^2 + (u_{ref})^2}$$

In which: U: expanded uncertainty, u: standard uncertainty, u_{Rw}= RSD_R standard deviation of internal reproducibility, u_{bias} was calculated from the recovery value and ignore the uncertainty u_{ref} of the standard used.

The result showed that measurement uncertainty of the method was 11.4 and 7.90% for ETO and 2-CE, respectively.

3.4. Sample analysis application

In these 1668 samples, there were 535 samples of noodles, 415 samples of spices, 168 samples of dried vegetable, 325 samples of oil seasoning and 225 samples of chili sauce. The results of the analysis are shown in Table 6.

Table 6. *Analysis result of 2-CE in 1668 samples*

Sample type	***Total (n)***	***Number of detected (n)***	***Detection frequency (%)***	***2-CE amount (mg/kg)***
Noodles	535	6	1.12	Trace - 2.38
Spices	415	41	9.88	Trace - 4.08
Dried vegetable	168	10	5.95	Trace - 486
Oil seasoning	325	16	4.92	Trace - 2.55
Chili sauce	225	5	2.22	Trace - 0.09
Total	1668	78	4.68	

Trace: less than LOQ level

Each sample was coded and homogenized before sending to the lab. ETO was not detected in all the analyzed samples. For 2-CE, 6/535 samples of noodles were detected from trace (less than LOQ level) to 2.38 mg/kg (1.12%), 41/415 samples of spices were detected from trace to 4.08 mg/kg (9.88%), 10/168 samples of dried vegetable were detected from trace to 486 mg/kg (5.95%), 16/325 samples of oil seasoning were detected from trace to 2.55 mg/kg (4.92%) and 5/225 samples of chili sauce were detected from trace to 0.09 mg/kg (2.22%); total 78/1668 samples were positive (4.68%).

This result was consistent with previous study conducted in Germany [23], 2-CE appeared in high concentration mainly in dried vegetable and spice samples. This may stem from the widespread use of ETO as a fumigant in agricultural products. All 2-CE positive noodle samples were pre-mixed with dried vegetables, samples without dried vegetables were not detected.

Therefore, the maximum residue limit is still not unified globally for ETO and its metabolites. On the other hand, although there are many potential risks to human health, up to now, Vietnam has not had regulations related to the allowable levels of ETO and its metabolites in food. The method has been designated as a method for state management in the field of food safety. The results of the study contribute to providing data for Vietnamese regulatory agencies in the future.

4. CONCLUSION

A sensitive method for analysis of ETO and its metabolite, 2-CE in food has been reported. The method was validated and meets the AOAC International requirements for selectivity, specificity with a good linear range of 0.01 ÷ 0.20 mg/kg. LOD and LOQ were 0.003 and 0.01 mg/kg. The recoveries of analytes were from 93.8 - 110.0% and the relative standard deviations were within the range of 1.90 ÷ 6.36 %. The measurement uncertainties were 11.4 - 7.90% for ETO and 2-CE, respectively. The method was qualified to conduct interlaboratory validation. It might become a standard method that will contributing to safety confirmation of ETO and 2-CE containing products.

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Phân tích ethylene oxide và 2-chloro-ethanol sử dụng sắc ký khí khối phổ hai lần: sử dụng nội chuẩn và bi thép không gỉ trong xử lý mẫu thực phẩm có hàm lượng chất béo cao

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Tóm tắt

Phương pháp sắc ký khí khối phổ hai lần (GC-MS/MS) đã được nghiên cứu và thẩm định nhằm xác định ethylene oxide (ETO) và 2-chloro-ethanol (2-CE) trong thực phẩm, đặc biệt là những thực phẩm có hàm lượng chất béo cao. Phương pháp tách được thực hiện qua cột TG-WAX (60 m × 0,32 mm × 1 µm), với kỹ thuật tiêm thể tích lớn và hóa hơi với chương trình nhiệt độ (PTV-LVI). ETO, 2-CE, và nội chuẩn đồng vị 2-chloro-ethanol-d4 (2-CE-d4) được xử lý bằng phương pháp QuOil (một biến thể của phương pháp QuEChERS), kết hợp với việc sử dụng bi thép không gỉ, đặc biệt hiệu quả với chất nền có hàm lượng chất béo cao và được phân tích bằng phương pháp sắc ký khí khối phổ (GC-MS/MS). Phương pháp này có độ đặc hiệu và độ chọn lọc cao với giới hạn phát hiện 0,003 mg/kg, khoảng tuyến tính 0,01 - 0,2 mg/kg, độ tái lập và độ thu hồi đáp ứng các yêu cầu của AOAC. Phương pháp này đã được áp dụng để phân tích 1668 mẫu thực phẩm trong giai đoạn 2021-2022 tại Viện Kiểm nghiệm an toàn vệ sinh thực phẩm Quốc gia (NIFC).

Từ khóa: *ethylene oxide, 2-chloro-ethanol, QuOil, bi thép không gỉ, GC-MS/MS.*