Determination of Ethylene Oxide Residues in Dairy Products and Ice Cream by Gas Chromatography-Mass Spectrometry

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Abstract: A novel quantitative method for the determination of ethylene oxide (EO) residues indairy products and ice cream by gas chromatography tandem mass spectrometry (GC-MS/MS) was established. The method was based on the "A-B" mode, and the external standard was used for quantitative analysis through the ethylene oxide matrix -fortified standard curve. In the presence of chloride ions, EO in tube A could be converted into its metabolite 2-chloroethanol (2-CE) by acid hydrolysis. Endogenous 2-CE was measured in tube B. Then the acetonitrile extractions from tube A and tube B were purified prior to instrumental analysis by QuEChERS. According to experimental data, the linear relationships of EO were good in the concentration range of 0.005~0.25 μg/mL and the correlation coefficients (R^2) were not less than 0.995. Meanwhile, recovery experiments expressed the recoveries from 77% to 109% and the range of relative standard deviation (RSD) was 3.88%-14.5% at the investigated levels (0.01, 0.02 and 0.10 mg/kg) with the limit of detection (LOD) at 0.005 mg/kg and limit of quantification (LOQ) at 0.01 mg/kg. The LOQ was low enough to ensure compliance with regulatory requirements, as well as accurate, repeatable, sensitivity to meet the detecting requirements for differentdairy products and ice cream, such as milk, fermented milk, milk powder and ice cream.

Keywords: Ethylene Oxide; 2-chloroethanol; GC-MS/MS; Dairy Products and Ice Cream; Residue

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1 Introduction

Since the autumn in 2020, the world's demand for ethylene oxide (EO) analysis had increased significantly with the recall or withdraw of contaminated dairy products and ice creams related to the presence of EO in a dairy products and ice cream additive such as locust bean gum (E410) in the European Commission (EC). As was known to all, EO was a gaseous disinfectant and it was usually used as pesticide and fumigation to control insects, fungi and bacterial pathogens in dry dairy products and ice cream products [1]. Due to its toxicity, highly carcinogenic, mutagenic and genotoxic impurity for living being, it was banned in the European Union (EU) as a pesticide in 1991 and as an output product indairy products and ice cream in 2011. According to the Commission Regulation 2015/868 [2], the maximum residue level (MRL) of EO and 2-CE from 0.02 to 0.1 mg/kg in different products. EO was classified as a

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category 1 carcinogen by the International Agency Research of Cancer (IARC) [3] and the United States regarded EO as a "known human carcinogen" by the Family Health Research Institute (FHRI) [4].

Due to its high volatility with small molecular weight (C_2H_2O, 44.05 g/mol) and low boiling point (10.4 ℃), it was reported that once in contact with thedairy products and ice cream, EO undergoed various reactions with the major matrix constituents leading to the formation of several metabolites, such as 2-CE, or dissipated through evaporation [5]. EO, 2-CE (Figure 1) and their various reaction products were only removed at a limited extend. Consequently, it was essential to detect the residues of EO and its metabolites. Owing to the toxicity of EO and 2-CE, a joint-residue definition for the two components was introduced in Regulation (EC) No 149/2008 [6] in 2008. To enforce these regulations, accurate analysis methods for these contaminants indairy products and ice cream were imperative.

Ethylene oxide (Molecular Formula-C_2H_6O)

2-Chloroethanol (Molecular Formula-C_2H_5ClO)

Figure 1 The structure and molecular formula of EO and 2-CE

Until now, multitudinous methods for the analysis of EO residue had been published and commodities focused on drydairy products and ice creams such as herbs, spices, dried vegetables, nuts, grain [7-11], instant noodles [12], or medical devices [13] which were inappropriate in dairy due to containing many interfering compounds such as protein, fat and phospholipid. The detection of the analytes was implemented usually by separation with gas chromatography (GC) coupled either with electron capture detection (ECD), flame ionisation detection (FID), or mass spectrometry (MS, single or triple quadrupole). These methods were classified mainly to direct measurement and indirect determination. The direct measurement was convenient and fast, but it depended on programmed-temperature-vaporization (PTV) injector or headspace injection, which had high requirements on equipment and poorly popularized in general laboratory. For example, a more authoritative and official method was developed that used QuEChERS extraction followed by GC/MS/MS analysis by European Commission in 2020 focusing primarily on spices, oily seeds, and nuts, but PTV programmed injection was required for this method [14]. Another method was proposed by The Environmental Protection Agency (EPA) which described the detection of EO and 2-CE in spices by headspace GC [15]. Considering the limitations of headspace injection and PTV injection, indirect determinations were popularized vigorously, which were based on the conversion of EO to 2-CE under acidic conditions in the presence of chloride ions [16] or the conversion of 2-CE to EO under alkaline conditions [17]. The calculation of EO was achieved by internal standard method based on the full conversion of EO to 2-CE which was reported in literatures [18]. But there was no suitable method to avoid EO losses during analysis required additional time-consuming and tedious precautions, so it was unbelievable for the full conversion of EO to 2-CE along with a overestimation of EO residues in external standard method. Simultaneously, a report in 2021 had validated that the yield of EO to 2-CE was merely 50% due to its high volatility and/or react under ambient conditions with nucleophiles such as chloride to form the less volatile metabolite 2-CE (boiling point 129 ℃) [4], which was resemble extremely to our results and conclusion. So it was not accurate enough to calculate the actual content of EO based on the theoretical conversion of EO to 2-CE. Therefore, the purpose of this study was to develop a novel quantitativemethod for EO by GC-MS/MS to solve the inaccurate quantification without considering actual yield of EO to 2-CE.

To our knowledge, this study was first to establish a simple and practical quantitativemethod based on external method by GC-MS/MS, which utilized “A-B” tube to quantify EO residue while considering the actual yeild of EO to 2-CE, where A represented the response of native EO and endogenous 2-CE, B represented the response of endogenous 2-CE which leaded to redundant response except native EO in A. The subtraction of A and B was the EO residue when quantified with EO-matrix-fortified standard. It provided insight into the distribution of the EO versus the marker compound 2-CE in dairy products.

2 Apparatus and Materials

2.1 Chemicals and Reagents

Unless otherwise specified, all chemicals and reagents were of reagent grade or higher. The solvents used were LC gradient grade. Ultra-pure water (Milli-Q Plus System, Millipore, 18.2 MΩ) was prepared in laboratory. Ready-to-use QuEChERS salts (containing 4 g of magnesium sulphate, 1 g of sodium chloride, 1 g of sodium citrate and 0.5 g of disodium citrate sesquihydrate) and dis-

persive Solid Phase Extraction salts (d-SPE, containing 150 mg PSA, 150 mg C18EC, and 900 mg $MgSO_4$) were obtained from Agilent.

Analytical standards of EO (50 mg/mL in methanol, ≥98% purity) and 2-CE (2 mg/mL in methanol, ≥98% purity) were acquired from Dr. E (Augsburg, Germany). According to actual demand, standards of EO and 2-CE were diluted into 1 μg/mL by acetonitrile. Due to the high volatility of EO, related standard solutions and acetonitrile should keep cooler and all operations need to be completed quickly. Stock solutions were stable at −20 ℃ for at least one month and working solutions should be prepared when using.

2.2 Reagent Preparation

2.2.1 Aqueous Sodium Chloride Solution (370 g/L)

37 g sodium chloride dissolved in 100 mL water and mixed by ultrasonic.

2.2.2 Aqueous Sulfuric Acid Solution (0.05 mol/L)

0.27 mL concentrated sulfuric acid dissolved in 100 mL water.

3 Experimental

3.1 Sample Extraction

The extraction of EO and the endogenous 2-CE were performed according to the reported method of ice cream from Bessaire et al [16] with minor modifications.

A tube: Each sample was weighed into a 50-mL polypropylene (PP) tube (A tube) and placed in an ice water bath. 5 mL water and 5 mL saturated NaCl solution (2.2.1) as well as 100 μL sulphuric acid solution (2.2.2) were added in sequence and the tube was vortexed for 1 min. The yield of EO to 2-CE was then conducted at 60 ℃ ± 2 ℃ for 120 min by placing the tube onto a horizontal shaking water bath. Once completed, the tube was rapidly cooled down in an ice water bath for 10 min.

Notes 1: milk, fermented milk and ice cream were weighed 5 g; milk powder and cheese were weighed 2.5 g.

B tube: For analysis of the endogenous 2-CE was operated without acid hydrolysis. Each sample was weighed into B tube (B tube was the same as A tube) and then water was added in the same tube. The volume of water will depended on the type of sample.

Notes 2: The weigh was consistent with tube A.

Notes 3: 5 mL water was added when analytes were milk fermented milk and ice cream. 10 mL water was added when analytes were milk powder as well as cheese. Additional water was redundant when analyte was milk.

Extraction and purification of 2-CE: Extraction and purification of 2-CE from tube A and B were further performed following the European Norm EN 15662: 2018 (QuEChERS-based procedure) [19] with subtle adjustments.

10 mL ACN was added to the tube and the resulting mixture was shaken thoroughly for 5 min using vortex mixer. The ready-to-use QuEChERS salts mixture were then added and the tube was immediately hand shaken to prevent the formation of lumps. After shaking (3 min) and centrifugation (15000 r/min, 0~4 ℃, 5 min), an aliquot of the supernatant was transferred into a 15-mL PP tube already filled with $MgSO_4$, PSA and C18 (d-SPE salts). After shaking (5 min) and centrifugation (15000 r/min, 0~4 ℃, 5 min), the supernatant was transferred into a new 15-mL PP tube and concentrated under a stream of nitrogen at room temperature to nearly dry. The concentrated extract was diluted to 1mL by ACN and further passed through organic microporous filter membrane. Then the final extract was collected into a GC amber glassvial for further GC-MS/MS analysis.

Notes 4: For the first transferred supernatant, milk, fermented milk and ice cream need 6 mL, milk powder and cheese need 7 mL.

Notes 5: For the second transferred supernatant, milk, fermented milk and ice cream need 2.5 mL, milk powder and cheese need 4 mL.

3.2 GC-MS/MS Methord

In this study, the performance of the Thermo Scientic 9000 triple quadrupole GC-MS/MS system equipped with the Electron Ionization (EI) source was evaluated for the analysis of EO in solvent standards and real sample. The samples were injected by a Thermo Scientific Trace 1310 GC with Split/Splitless (S/SL) injector. The acquisition and processing of data were carried out with Thermo Scientific Chromeleon 7.3.1 and TraceFounder 4.1. respectively.

3.2.1 Chromatographic Conditions

For the analysis of 2-CE after extraction techniques, a

30 m × 0.25 mm, 0.25 μm DB-Wax capillary column was used. A 1 μL volume was injected in splitless mode with injector temperature at 200 ℃. The oven temperature programme was as follows: 45 ℃ (2 min), 50 ℃/min to 150 ℃, 70 ℃/min to 240 ℃ (post run at 240 ℃ for 10 min).

3.2.2 Mass Conditions

Triple quadrupole was operated in selective reaction monitoring (SRM) mode and collision energy were 5 eV (Figure 2). The temperatures of the transfer line, ion source were 230 ℃ and 230 ℃, respectively. The collision cell gases and carrier gas were Argon (1.5 mL/min) and Helium (1.0 mL/min), respectively. Ion transitions and retention time of 2-CE as shown in the table 1.

Table 1 Ion transitions and retention time of 2-CE

Compound	Retention time (min)	Ion transitions (m/z)
	5.30	80/31
2-CE	5.30	80/43
	5.30	82/31

3.3 Matrix-fortified Standard

Accurately pipette stock slution of EO (1.00 μg/mL) to prepare calibration solutions with concentrations of 5, 10, 20, 50, 100 and 150 ng/mL. Then 1.0 mL calibration solution at different concentrations was added in blank samples with the same matrix, respectively. Pretreatment and purification were implemented according to the procedures previously mentioned.

3.4 Qualitative Analysis

The molecule 2-CE was considered as positively identified in the sample when the following criteria were fulfilled as required by SANTE/11312/2021 [20] and (EU) 2021/808 [21].

1) a signal was visible at least at two diagnostic transition reactions selected for 2-CE.
2) the retention time of 2-CE in the sample extract corresponded to that of the average of the matrix-fortified standard measured in the same sequence with a tolerance of ±0.1 min.
3) peak area ratio from the different transition reactions recorded for 2-CE in the sample extract should correspond to that of the average of the calibration solutions measured in the same sequence with a tolerance as shown in the table 2.

Table 2 Maximum allowable deviation of relative abundance of qualitative ions for analytes

peak area ratio	>50%	>20%~50%	>10%~20%	≤ 10%
Relative deviation.	±10%	±15%	±20%	±40%

3.5 Quantitative Analysis

The content of EO in sample was calculated as follows:

$$x_i = \frac{(C_{Ai} - C_{A0}) \times V \times F}{m_A \times 1000} - \frac{(C_{Bi} - C_{B0}) \times V \times F}{m_B \times 1000} \quad (1)$$

Where $C_{A,i}$ was the concentration of analyte in the sample in tube A, C_{A0} was the concentration of analyte in blank in tube A, m_A was the mass of the test portion in tube A. C_{Bi} was the concentration of analyte in the sample in tube B, C_{B0} was the concentration of analyte in blank in tube B, m_B was the mass of the test portion in tube B. *1000* was the yield factor to express concentration from μg/kg to mg/kg. V was the constant volume. F was the dilution factor.

3.6 Statistical Analysis

The data of optimization and verification were analysed by Microsoft Excel 2021 Software.

4 Results and Discussion

4.1 Optimization of GC-MS/MS Conditions

At present, the reported methods mainly focused on multi-mode injection ports, which had high requirements for equipment. By replacing multi-mode injector with split/splitless (S/SL) injector, the requirements for instruments were reduced and it was easy to be promoted. 2-CE was a polar compound with high boiling point (128.8 ℃) and strong volatility, the split injection was selected couple with optimizing the injector temperature and injection volume. Nevertheles, the 2-CE peak was poor, the response and sensitivity were lower. When splitless injection mode was selected, the peak and response as well as

sensitivity were improved. According to the Korean standard [22], the temperature of the injector was set at 200 ℃ and the injection volume was 1 μl, respectively. Fortunately, the peak shape and sensitivity were consistent with the expected effect, as shown in Figure 2.

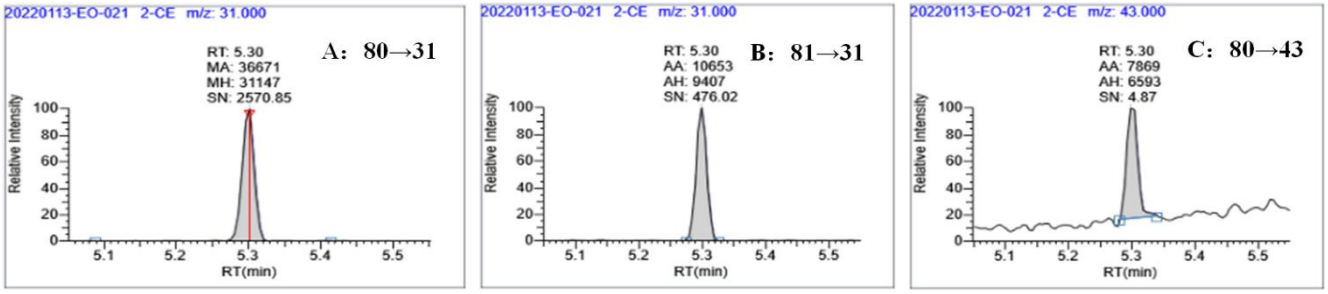

Figure 2 Ion transitions of 2-CE standards at 50 ng/mL

4.2 Yield Validation

Due to the high volatilityof EO, it was a high challenge for the management of standard solutions and fortification experiments. In order to verify the changes of yield of EO to 2-CE with the conversion temperature, conversion time, sulfuric acid dosage as well as the EO concentration, some recovery experiments were conducted. The results indicated that the yield was 50% approximately after optimizing different factors which was consistent with the reported literature [3].

4.2.1 Effect of Conversion Temperature

Temperature was a significant factor in conversion of EO to 2-CE. When the concentration and time were constant, increasing the temperature would increase the energy of reactant molecules and increase the number of effective collisions, thus reaction rate was increased. Different temperatures ranging 10 ℃ to 80 ℃ with a 10 ℃ interval were verificated in Figure 3. As displayed in Figure 3, When the conversion temperature was 10 ℃, the yield was less than 20%. With the increase of temperature, the yield was increased to 53% when the temperature reached 60 ℃. However, continue to increase the temperature, the yield was gradually decreased and returned to 43% when the temperature rised to 80 ℃. So the preferred conversion temperature was 60 ℃.

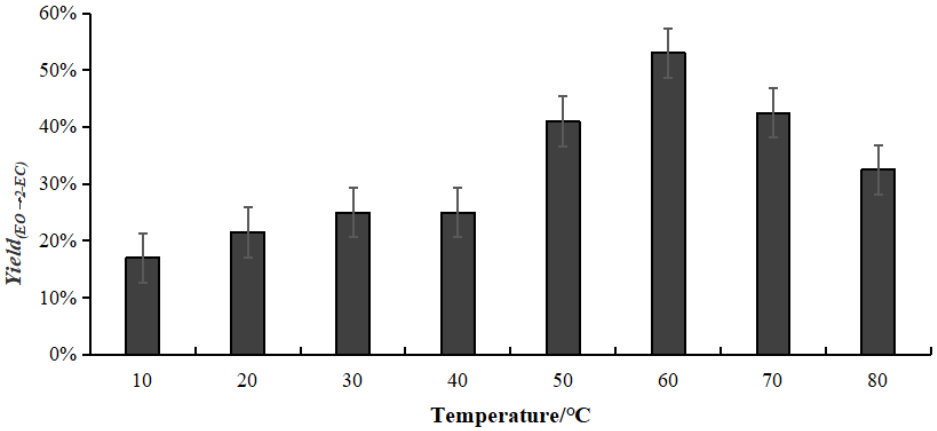

Figure 3 Effect of temperature on yield of EO to 2-CE

4.2.2 Effect of Conversion Time

Generally speaking, as time going on, the reactant concentration would decrease. The reaction rate would slow down until the chemical equilibrium was reached. The change of yield with the increase of conversion time was shown in the figure 4. In the range of 0.5-2 h, the yield gradually increased from 21% to 52%. What's more, there was no obvious changes in yield with time continued increasing. So the proper conversion time was 2 h.

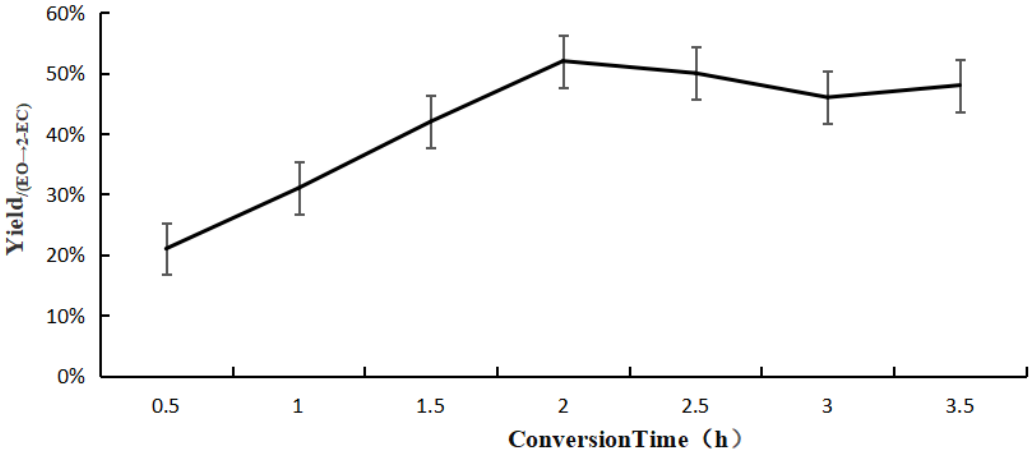

Figure 4 Effect of time on yield of EO to 2-CE

4.2.3. Effect of Sulfuric Acid Dosage

Under the condition of the optimized conversion temperature (60 ℃) and time (2 h), the effect of dosage of acid (0.05 mol/L) on the yield was depicted in Figure 5. In the range of 20-100 μL, the yield gradually increased from 13% to 54%. However, the yield tended to decline when acid dosage increasing from 100 μL to 250 μL. So the compatible acid dosage was 100 μL.

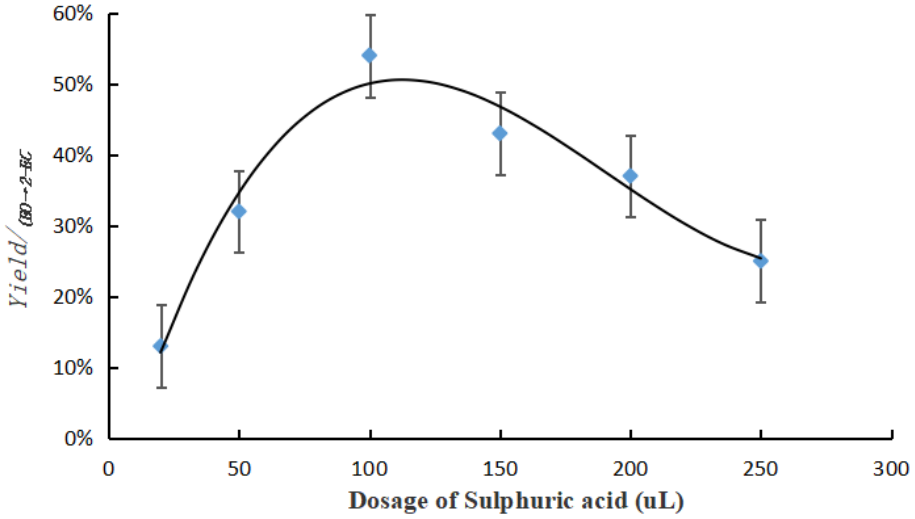

Figure 5 Effect of acid dosage on yield of EO to 2-CE

4.2.4 Effect of EO Concentration

After optimizing conversion temperature (60 ℃), time (2 h) and acid dosage (0.05 mol/L, 100 μL), the effect of EO concentration on yield should be investigated. After acid hydrolysis, the different concentrations expressed as 2-EO equivalent in the standard solutions (C_{EO}, expressed in ng/mL) were calculated as follows:

$$C_{2-CE}=C_{EO}\times\frac{M_{2-CE}}{M_{EO}}$$

where $M_{2\text{-}CE}$ and M_{EO} were molecular masses of 2-CE (80.52 g/mol) and EO (44.05 g/mol), respectively. It was worth mentioning that there was no noticeable variation in yield with EO concentration changed, as indicated in Figure 6.

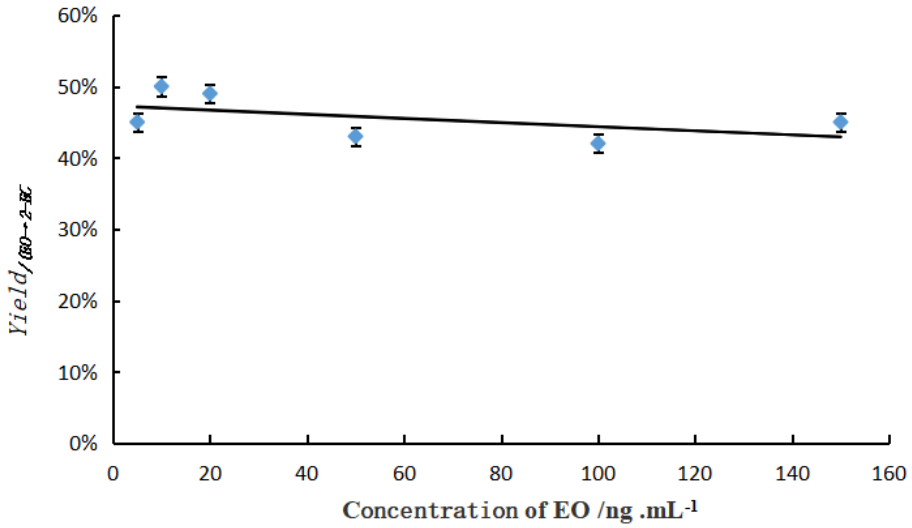

Figure 6 Effect of EO concentration on yield of EO to 2-CE

4.3 Quantitative Curve

The content of endogenous 2-CE affect seriously the choose of quantitativemethod for EO. If there was no endogenous 2-CE, it was unnecessary to establish tube B, and EO could be measured directly through tube A with EO calibration curve. Whereas, once endogenous 2-CE existed, tube B was particularly indispensable and the selection of proper quantitativemethod was also crucial. In order to emphasize the importance of quantitativemethod, some fortificated experiments were implemented and four quantitativemethods were proposed to demonstrate the effect of different curves on the quantitative results in milk. The four schemes included the following:

1) The quantitativemethod was EO-matrix-fortified calibration curve in tube A and B.
2) The quantitativemethod was 2-CE calibration curve in tube A and B.
3) The quantitativemethods were 2-CE calibration curve and EO-matrix-fortified calibration curve in tube A and tubeB, respectively.
4) The quantitativemethod was EO-matrix-fortified calibration curve in tube A and the quantitativemethod was 2-CE calibration curve in tube B.

As demonstrated in figure 7, the recoveries at different levels were less than 60% if scheme (b) and scheme (c) were adopted explained by the yield of EO to 2-CE was less than 100% instead of matrix effect (Table 3). However, when scheme (d) was used to calculate the concentration of EO in sample, the recoveries were more than 120%. Nothing other than scheme (a) was employed and the recoveries were in a acceptable range (60%-120%). In order to make results accurate avoiding false guidance, the quantitativemethod including EO-matrix-fortified calibration curve were favoured in tube A and B.

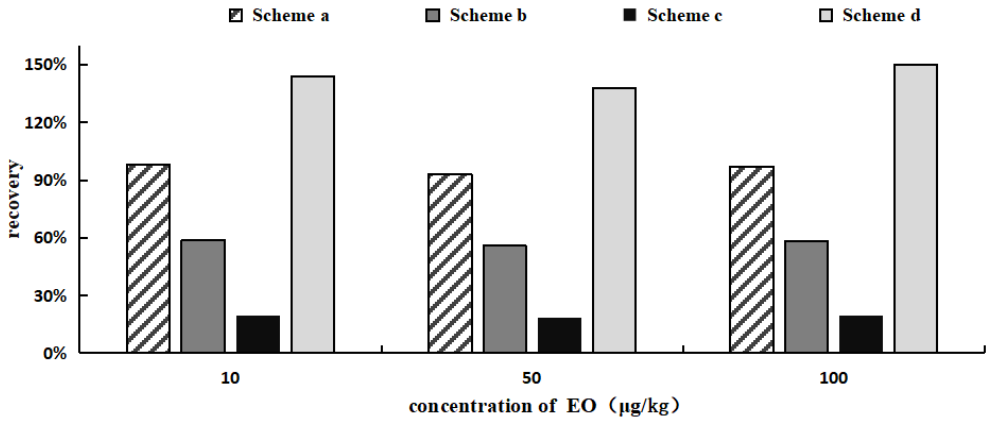

Figure 7 Effect of different curve on the recovery of EO in milk at different levels

Table 3 Matrix effect of 2-CE in different dairies

Commodities	Matrix fortified calibration curve		solvent calibration curve		matrix effect (ME)
	Slope	R2	Slope	R2	
milk	458.73	0.9999	427.67	0.9991	107%
fermented milk	382.84	1.0000	384.79	0.9973	99%
ice cream	361.96	0.9999	381.35	0.9978	95%
powdered milk	303.9	0.9998	329.05	0.9997	92%

Meanwhile, the effect of different curves on the quantitative results was validated in different dairies. Only when EO-matrix-fortified was selected, the recoveries were within the range of 60%-120% and the results were consistent with the tendency in milk. So EO-matrix-fortified calibration curve was choosen for the quantitative analysis of EO avoiding erroneous results.

4.4 LOD, LOQ and Linear Regression

In the light of SANTE/11312/2021 document (European Commission 2021) [20], the limit of determination (LOD) was the validated lowest residue concentration which could be quantified and reported by routine monitoring with validated control methods, but the limit of quantification (LOQ) was defined as the lowest validated levels with acceptable accuracy. The lowest concentration corresponding to the signal to noise (S/N) more than 3 was regarded as LOD, LOQ was determined by taking the lowest concentration of EO in the linear range along with recovery in the range of 60%~120% (figure 4), which were set at 0.005 mg/kg and 0.010 mg/kg, respectively.

The different matrix-matched standard working solutions were measured according to the procedures in 3.1, matrix-fortified standard curve were established as shown in Table 4. The linear range of the matrix fortified calibration curve was 5~150 ng/mL followed by a linear method with $R^2 > 0.99$ and the deviation of the back calculated concentrations were < 5% for each individual calibration point indicating excellent suitability.

Table 4 Linear regression equation, LOD and LOQ

Commodities	Target	linear range	matrix-fortified calibration curve	LOD (mg/kg)	LOQ (mg/kg)
milk	EO	5~150 mg/kg	$Y = 361.1X + 1466$, $R^2 = 0.9981$	0.005	0.01
fermented milk			$Y = 355.07X - 64.145$, $R^2 = 0.9988$	0.005	0.01
ice cream			$Y = 324.51Y + 95.971$, $R^2 = 0.9992$	0.005	0.01
powdered milk			$Y = 314.42Y + 3774$, $R^2 = 0.9992$	0.005	0.01

4.5 Accuracy and Precision

A lower number of blank samples (n = 72) were analysed after fortification with EO at study levels (0.010, 0.020, 0.100 mg/kg). Accuracy was expressed as recovery and precision was expressed in terms of relative standard deviations (RSD). As revealed in Table 5, the recoveries of EO were from 78% to 109% along with a within-laboratory precision between 3.9% and 14.5%, irrespective of the dairy products and ice cream commodity group and fortification level. Significantly, it demonstrated that not EO, but 2-CE was the predominant analyte detected in the contaminated samples. What's more, the potential capability of the method was proved to detect and quantify EO in dairy categories at different levels.

Table 5 Results of EO fortification experiments in 72 dairies *(n = 6)*

commodities	EO fortification levels (mg/kg)					
	0.01		0.02		0.1	
	Average recovery (%)	RSD (%)	Average recovery (%)	RSD (%)	Average recovery (%)	RSD (%)
milk	78	13.5	92	9.3	104	7.0
fermented milk	90	10.8	99	4.5	109	7.9
ice cream	81	5.5	84	3.9	96	6.0
powdered milk	89	14.5	98	9.4	101	5.6

4.6 Sample Detection

To demonstrate the accuracy of the novel quantitative method based on "A-B" method and GC-MS/MS, 26 representative samples collected in a local market were analyzed. None of EO was detected in the samples, probably explained by the samples which suffered losses during production, storage or processing where the volatilization of EO was difficult to control.

5 Conclusion

This study was first to establish a creative quantitative method and quantify with EO -matrix-fortified calibration. In such perspective, conversion of EO into 2-CE was essential enough when samples already contain high levels of 2-CE, leading to erroneous results. An innovative quantitative method based on "A-B " method combined with acid hydrolysis in the presence of chloride ion followed by QuEChERS sampling technology and GC-MS/MS analysis was established. All the performance characteristics were in line with guidance document DG SANTE/12682/2021 recommendations. The method provided excellent sensitivity, good reproducibility and wider linear range (5-150 ng•mL^{-1}) with correlation coefficient R^2>0.99. The LOQs (0.01 mg/kg) were, in all cases, significantly lower than the MRLs from Regulation [EC] NO 396/2005. The proposed method not only could be adapted to EO residues in other dairy (e.g cheese) with more accuracy and better performance, but also had great potential to alter the current conventional quantitative method for routine analysis of EO residues with standard curve of 2-CE. Finally, this study confirmed the absence of native EO in dairy samples.

Conflicts of Interest

No potential conflict of interest was reported by the author(s).

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