



Development of a new dispersive liquid-liquid microextraction method to ensure the conformity of cachaça for ethyl carbamate by GCxGC/FID

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ABSTRACT

Ethyl carbamate (EC) is a contaminant in fermented and distilled beverages, and its presence in cachaça raises an economic and health concern. A methodology for the analysis of EC in cachaça was developed, combining the new extraction method, dilution-assisted dispersive liquid-liquid microextraction (DA-DLLME), with comprehensive two-dimensional gas chromatography with flame ionization detection (GC × GC/FID). This method aims to minimize toxic solvents while ensuring reliable distilled beverage analysis. A fractional factorial design determined the optimal extraction conditions, and statistically significant interactions between salt and solvent were observed in the extraction method. The method showed robust analytical performance with a limit of detection (LOD) of 37.52 µg L⁻¹ and a limit of quantification (LOQ) of 125.07 µg L⁻¹. Linearity was achieved with a correlation coefficient (R²) of 0.9703 in a range of 145.00–415.00 µg L⁻¹. Precision was 8.87% on average for intra-day and 8.64% for inter-day assays, indicating excellent repeatability and reproducibility. The extraction recovery ranged from 102.00% to 109.00%, and no matrix effect was observed. The method was applied to the analysis of cachaça samples and yielded EC concentrations of more than 415.00 µg L⁻¹. Therefore, the method is suitable for routine quality control. The DA-DLLME method offers a fast, efficient extraction with high enrichment factors. The new method and GCxGC enabled FID to detect EC at levels below the current legal limits. It is a promising tool for monitoring EC levels in distilled beverages and ensuring consumer safety and regulatory compliance.

1. Introduction

The detection of ethyl carbamate (EC) in fermented and distilled beverages, such as cachaça, has become a significant concern due to its carcinogenic potential. Primarily formed through the reaction of urea or cyanate with ethanol during fermentation (Abt et al., 2021), EC raises serious food safety and regulatory compliance concerns when present at elevated levels in alcoholic beverages. Additionally, its toxicological profile has drawn significant attention, particularly its presence in cachaça (Lachenmeier et al., 2010) and other distilled spirits. Cachaça is a sugar cane spirit with a geographical indication protected by the Brazilian government with regulations that define the specifics of its production and the standards for its chemical profile (Ratkovich et al., 2023). It is Brazil's most culturally and economically significant spirit.

According to official reports (Brasil, 2022a), approximately 7.2 million liters of cachaça were exported to 67 countries in 2021, with Paraguay, Germany, and the USA being the leading importers. Under Brazilian regulations (Brasil, 2022b; Ratkovich et al., 2023), the alcohol content of cachaça must be between 38 and 48% by volume. Limits are also set for organic and inorganic contaminants such as methanol (200 mg L⁻¹), acrolein (50 mg L⁻¹), *sec*-butyl alcohol (100 mg L⁻¹), *n*-butyl alcohol (30 mg L⁻¹), copper, and EC (210 µg L⁻¹).

The International Agency for Research on Cancer (IARC), Working Group on the Evaluation of Carcinogenic Risks to Humans (2010) classified EC as a group 2A carcinogen since there is sufficient evidence of carcinogenicity in experimental animals. Models suggest that EC is metabolized in the body after ingestion, leading to the formation of vinyl carbamate epoxide, the primary metabolite of EC. This molecule induces

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carcinogenicity and genotoxicity by forming DNA adducts, subsequently leading to mutations (Gowd et al., 2018). Canada was the first country to regulate the limits for EC content in beverages ($150 \mu\text{g L}^{-1}$), followed by the Czech Republic ($150 \mu\text{g L}^{-1}$), France ($150 \mu\text{g L}^{-1}$), and the United States ($125 \mu\text{g L}^{-1}$) (Abt et al., 2021; Chen et al., 2022).

Due to the quality control related to EC detection, analytical development is a critical step in its determination. It ensures accurate and sensitive methods that comply with regulatory standards. Traditional extraction methods such as Liquid-liquid extraction (LLE), solid-phase extraction (SPE), and solid-phase microextraction (SPME) have been used for EC extraction in beverages (Gowd et al., 2018). These conventional techniques have several limitations, including the need for large volumes of organic solvents, long extraction times, and often large sample sizes, which hinder miniaturization (Kannouma et al., 2022). In response to these limitations, dispersive liquid-liquid microextraction (DLLME) has emerged as a promising alternative for EC analysis. Offering rapid extraction and high enrichment factors, DLLME consists of a ternary mixture typically composed of an aqueous sample solution, an immiscible extraction solvent, and a disperser solvent (Ahmad et al., 2015). The disperser solvent function allows the distribution of the extraction solvent, creating a turbid mixture of fine droplets of the organic phase within the aqueous solution containing the analyte. El-Deen et al. (2023) reviewed traditional and innovative dispersion techniques for DLLME, highlighting their potential benefits for greener chemistry. They described dispersion by injection of extraction solvent, vortex stirring, vertical shaking, air bubbling, sonication, solid-based dispersion, and temperature-controlled dispersion.

Chlorinated solvents are commonly used as extraction solvents because of their immiscibility, with the aqueous donor phase and high density facilitating easy separation after centrifugation (Sajid, 2022). When a disperser solvent is sprayed into the donor phase, it can form a cloudy mixture and must have an affinity toward target compounds (Sajid, 2022). Parente et al. (2022) developed and validated an inverse DLLME, reporting that the order of injection, sample over solvents, allowed a better interaction of extraction solvent with aqueous samples. Kocúrová et al. (2010) explored the applications of a quaternary system to benefit the extraction solvent recovery after adding an auxiliary solvent. In the present work, the dispersion was formed by shifting the proportions of solvents in a water-ethanol-chloroform ternary system.

This paper presents a modified DLLME method, termed dilution-assisted dispersive liquid-liquid microextraction (DA-DLLME), developed to ensure compliance of cachaça samples with ethyl carbamate regulations. Additionally, it introduces the novel application of comprehensive two-dimensional gas chromatography coupled with flame ionization detection (GC \times GC/FID) for accurate measurement of EC.

2. Materials and methods

2.1. Reagents and solvents

Anhydrous disodium hydrogen phosphate, methyl carbamate (internal standard), and ethyl carbamate analytical standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). ACS grade sodium dihydrogen phosphate dihydrate and organic solvents ACS grade chloroform, HPLC grade ethanol, HPLC grade methanol were purchased from Merck (Darmstadt, Germany); sodium chloride was purchased from Synth (São Paulo, Brazil), and ultrapure water was obtained in a Micropure UV purification system from Thermo (Vantaa, Finland).

2.2. Samples collection

To evaluate the methodology performance, ten bottles of cachaça with an alcohol content of between 38.00% and 48.00% were purchased in the local trade of Minas Gerais, Brazil.

2.3. Alcohol content determination

To ensure the precision of measurements of ethanol content in cachaça samples and standard solutions, high-performance liquid chromatography with refractive index detector (HPLC/RID) method was developed and validated based on the method of Avila et al. (2018). The data and details can be found as supplementary material.

2.4. GCxGC/FID analysis

The measurement of EC was performed on an Agilent Technologies 8890 gas chromatograph configured for use in comprehensive two-dimensional gas chromatography, arranging in the first dimension (1D) a DB-5MS Agilent capillary column ($20.0 \text{ m} \times 0.180 \text{ mm} \times 0.18 \mu\text{m}$), in the second dimension (2D) a DB-17HT Agilent capillary column ($5.0 \text{ m} \times 0.250 \text{ mm} \times 0.15 \mu\text{m}$) and a FID detector. Hydrogen was used as carrier gas with a flow rate of 8.50 mL min^{-1} , injector temperature of 250.0°C , splitless injection mode, and injection volume of $1.00 \mu\text{L}$ using an Agilent 7693A Automatic Liquid Sampler. The oven was programmed with an initial temperature of 45.0°C held for 1.00 min, increasing by $8.0^\circ\text{C min}^{-1}$ to 100.0°C held for 1.00 min, increasing $50.0^\circ\text{C min}^{-1}$ to 290.0°C held for 1.3 min. The FID detector was set with a hydrogen flow of $40.00 \text{ mL min}^{-1}$ and a synthetic air flow of $450.00 \text{ mL min}^{-1}$, with nitrogen as make-up gas with a flow of $30.00 \text{ mL min}^{-1}$ and a flame temperature of 300.0°C . The system was equipped with an Agilent G4566A Reverse Fill/Flush CFT GC \times GC Differential Flow Modulator with a modulation time of 3.500 s and an injection time of 0.200 s.

2.5. Optimization of extraction parameters

The optimal conditions for extracting EC from cachaça were studied using multivariate optimization through a 2^3 factorial design with a triplicate of the central point. The diluent volume ($4.00\text{--}8.00 \text{ mL}$), salt concentration ($0\text{--}10.00\% \text{ wv}^{-1}$), and extraction solvent volume ($135.0\text{--}255.0 \mu\text{L}$) were the variables evaluated. The response variable was EC's GCxGC/FID signal extracted from the matrix. The centrifugation time (2 min at 4000 rpm), alcohol content ($38.30\% \text{ v}^{-1}$), and sample volume (8.00 mL) were kept constant. The volume of the blob of EC GCxGC/FID signal was the analytical response evaluated.

2.6. Sample preparation procedure

The dilution-assisted dispersive liquid-liquid microextraction (Fig. 1) was performed in a centrifuge tube. Initially, $135.0 \mu\text{L}$ of chloroform was added using a micropipette. An 8.00 mL sample followed this addition. For the samples were used an EC and methyl carbamate (MC) standard in ethanol ($38.3\% \text{ v}^{-1}$) or cachaça previously adjusted with ultrapure water to reach $38.3\% \text{ (v}^{-1}\text{)}$ of alcohol content. This adjustment was based on the data obtained from prior HPLC/RID analysis. The dispersion was performed by rapidly adding 8.00 mL of the phosphate buffer solution ($\text{pH} = 7.2 \pm 0.1$). The dispersed mixture was then centrifuged for 1.02 min at 4000 rpm, and the chloroform containing the substances of interest deposited in the bottom of the tube was collected and sent to GCxGC/FID analysis. The illustrated flowchart of the DA-DLLME procedure is provided as supplementary material. The relative retention time of EC to MC retention time allowed the identification of the EC GCxGC/FID signal.

2.7. Method validation

An analytical method validation procedure was applied to the developed method following the Eurachem guide (Magnusson and Ornemark, 2014). The linear regression for the internal standard calibration curve was expressed by the equation $y = a + bx$, where y is the chromatographic blob volume, and x is the concentration of EC. Being $S_{y/x}$ the standard deviation of the regression, the LOD and LOQ were

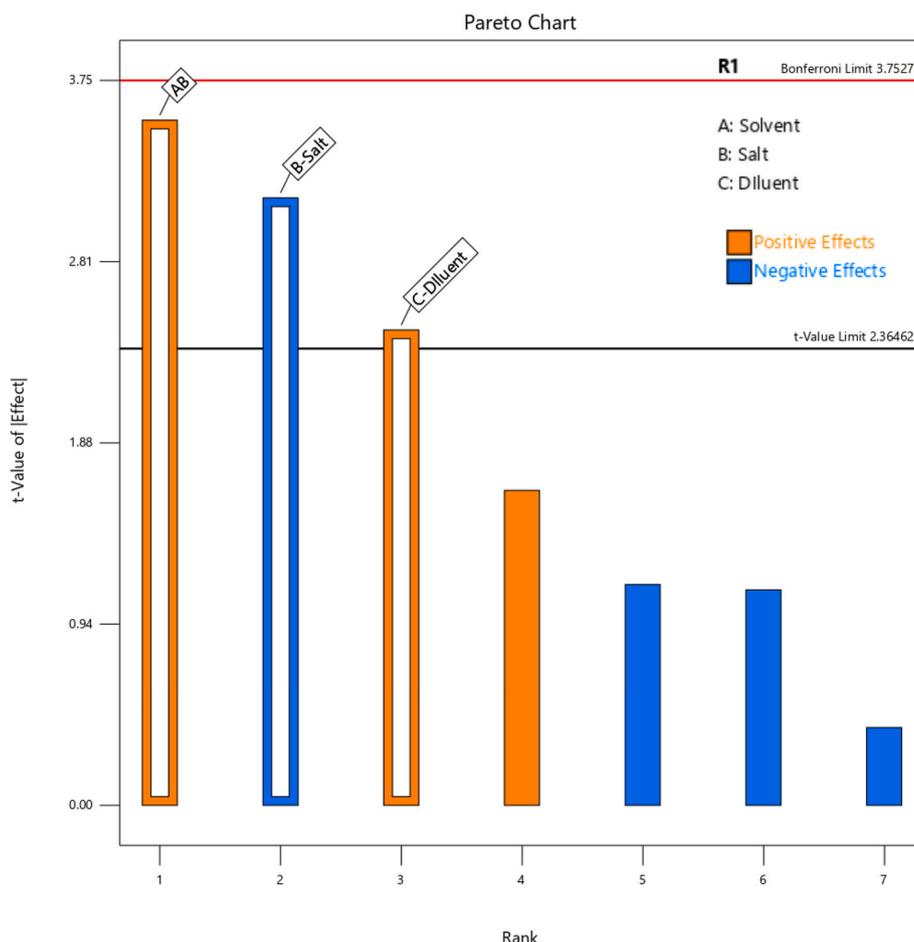


Fig. 1. Pareto chart of the factorial design for EC extraction by DA-DLLME-GCxGC/FID.

calculated as $3 \cdot S_{y/x}/b$ and $10 \cdot S_{y/x}/b$, respectively. Repeatability was measured by intra-day precision testing of six solutions of three concentration levels ($n = 6$). The inter-day tests were performed after seven days with the same three concentration levels ($n = 12$). The results were expressed in terms of the coefficient of variation (RSD %). The recovery was determined and expressed as a percentage of the quantity of the substance measured during the intra-day tests. The recovery was calculated relative to the concentration of the working solution (expected measured value) and was considered acceptable if it ranged from 80.0% to 120.0% (Oliveira et al., 2022).

The calibration graph was obtained using hydroalcoholic solutions of EC with concentrations of 145.00, 190.00, 235.00, 280.00, 325.00, 370.00, and 415.00 $\mu\text{g L}^{-1}$ ($n = 3$). For repeatability (intra-day, $n = 6$), intermediate precision (inter-day, $n = 12$), and recovery ($n = 6$), the levels of 190.00, 280.00, and 370.00 $\mu\text{g L}^{-1}$ were tested. The matrix effect was evaluated by an internal calibration (Magnusson and Örnemark, 2014). The solutions were prepared in a 25 mL volumetric flask containing 2.00 mL of cachaça previously adjusted with ultrapure water to an alcohol content of 38.30%. The EC and MC standards were added, and the volume was completed with ultrapure water. The homogeneity of the variance of the residuals of the internal and external calibration curves was assessed with an F-test, and a t -test was used to compare the slopes of the two curves.

3. Results and discussion

3.1. Optimization of extraction parameters

A 1.00 L of ethanol solution was prepared to be used as solvent for

the optimization and validation procedures. Using a Gay-Lussac scale alcoholmeter, the ethanol concentration of the solution was set to 38.00% vv^{-1} ethanol. According to the regulatory agencies, this concentration was chosen because it has the lowest possible value for ethanol concentration in cachaças. To ensure a greater precision of the value provided by the previous densitometric method, an aliquot of this solution was then analyzed via HPLC/RID. The chromatographic analysis results led to a more accurate quantification of ethanol in the solution and showed a concentration of 38.30% vv^{-1} . Since the optimization would be carried over with this solution, a precise adjustment to 38.00% vv^{-1} for the solvent was not considered necessary since the cachaça samples would be adjusted to the optimized ethanol concentration of 38.30% vv^{-1} .

This study identified the optimal extraction conditions through a two-stage multivariate approach. Initially, a fractional factorial design was applied, followed by a Doehlert design incorporating response surface methodology to refine the process further. The results of the fractional factorial design were analyzed using a Pareto chart (Fig. 2), which shows three significant variables. The positive interaction between salt and solvent showed that working with salt and solvent at the same level could increase the response value. That means it is possible to obtain higher analytical responses with 10.00% vv^{-1} salt solution, 255.00 μL of chloroform with no salt, and 135.00 μL of chloroform. It was opted to work with less solvent and no salt. The optimum condition for the maximum analytical response predicted by the factorial model was derived from the surface plot (Fig. 3). The optimized extraction conditions were 135.00 μL of chloroform, no NaCl addition, and 8.00 mL of diluent for 8.00 mL of sample.

Although the literature suggests that using NaCl could enhance the

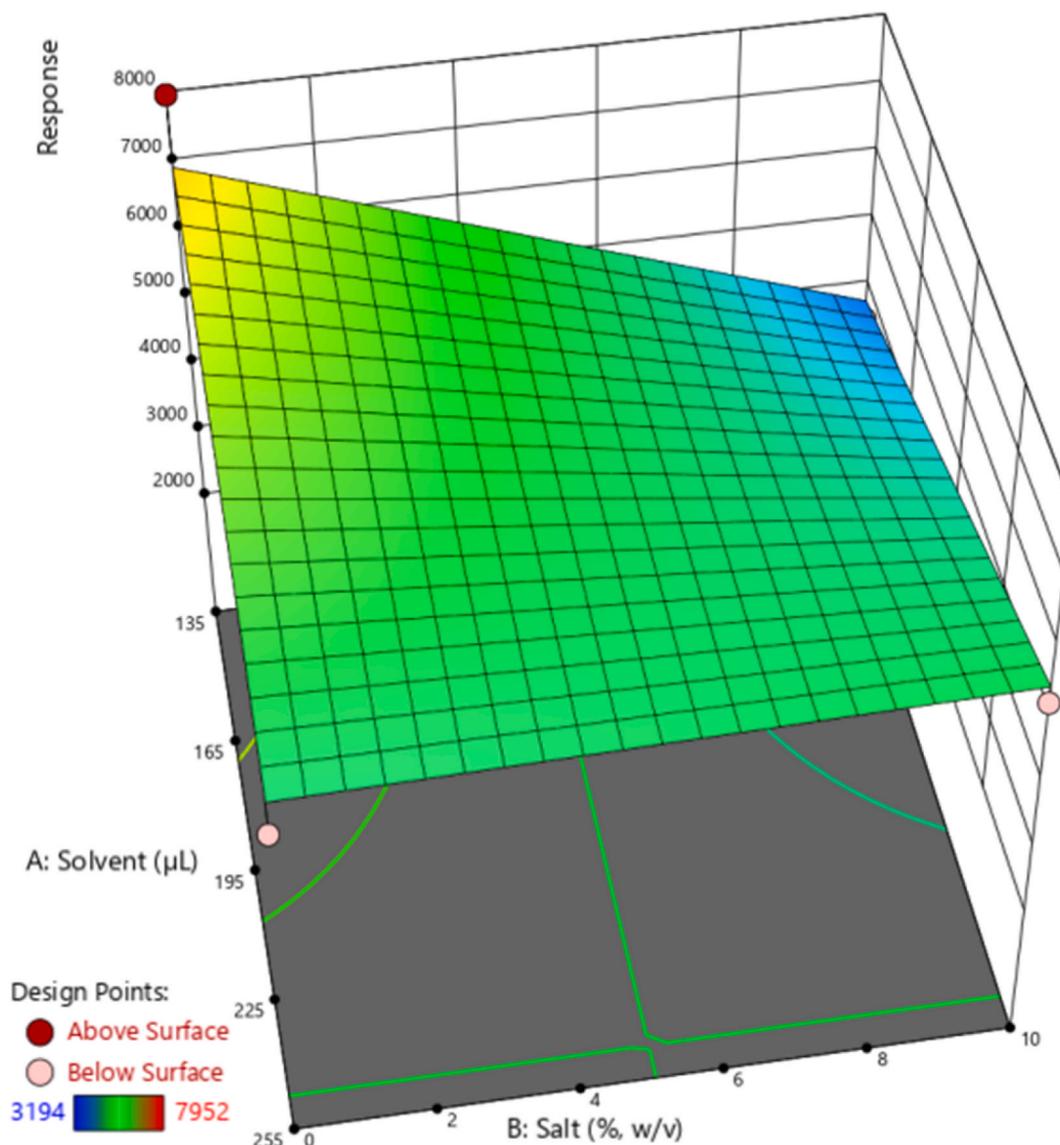


Fig. 2. 3D plot of the response surface for the factorial design, set at the level of 8.00 mL of diluent.

EC analytical response, the Pareto chart reveals a contrasting result. An increased salt concentration reduced the GCxGC/FID signal for EC, likely due to the unique mobility of EC in liquid-liquid and solid-phase extraction. Probably due to the peculiarities of EC mobility in liquid-liquid extraction, it was not possible to see the positive effects of NaCl reported by Machado et al. (2013) for the solid-phase extraction of EC in cachaça. Liao et al. (2013) investigated EC extraction in wine. They reported that the NaCl addition increased the EC analytical response, but this effect was not observed in the present optimization for DA-DLLME. In the DA-DLLME method, NaCl may hinder EC migration into the extraction solvent when micelles are formed due to the electronic double-layer repulsion effect, as Rezaie et al. (2023) discussed in their study on salts and emulsions. Thus, adding NaCl is not beneficial in this context, noting that no NaCl and lower level chloroform concentrations are optimal for enhancing EC detection.

The volume of the diluent was also significant. Adding 8.00 mL of diluent lowered the alcohol content to 19.15%. When less than 8.00 mL was added, an increase in the total volume of chloroform recovered was observed after breaking the emulsion by centrifugation. The increase in recovered volume is likely related to decreased EC response at higher alcohol content, as this may dilute the extract and reduce the enrichment factor. A similar observation was reported for EC analysis in wine

using ultrasound-assisted microextraction, finding the optimal alcohol content was 20% (Liao et al., 2013). The significant interaction between solvent and salt led to the choice of using the lowest volume of chloroform, while no NaCl was used in the dilution solution. In addition to increasing the analytical signal, using fewer reagents contributed to developing a greener method. Therefore, the dilution of the samples increased extraction efficiency with minimal solvent use.

The choice of a dispersive liquid-liquid microextraction technique for the sample preparation step preceding GCxGC/FID analysis was influenced by the presence of ethanol in the cachaça matrix, which serves as a natural disperser solvent. DLLME is a widely used sample preparation method for analyzing beverages (El-Deen et al., 2023). DLLME, followed by GC-MS, has been selected to analyze polycyclic aromatic hydrocarbons in cachaça (Will et al., 2018).

Considering the magnitude of EC concentration in cachaça (micrograms per liter), a large sample volume was necessary to achieve a higher enrichment factor. However, it was noted that the sample could dilute small volumes of the extraction solvent due to the volume of ethanol in its composition. The observed solubility of the extraction solvent leads to two possible extraction method approaches: increasing the volume of the extraction solvent or decreasing the alcohol content of the sample. To avoid using larger amounts of toxic solvents, it was

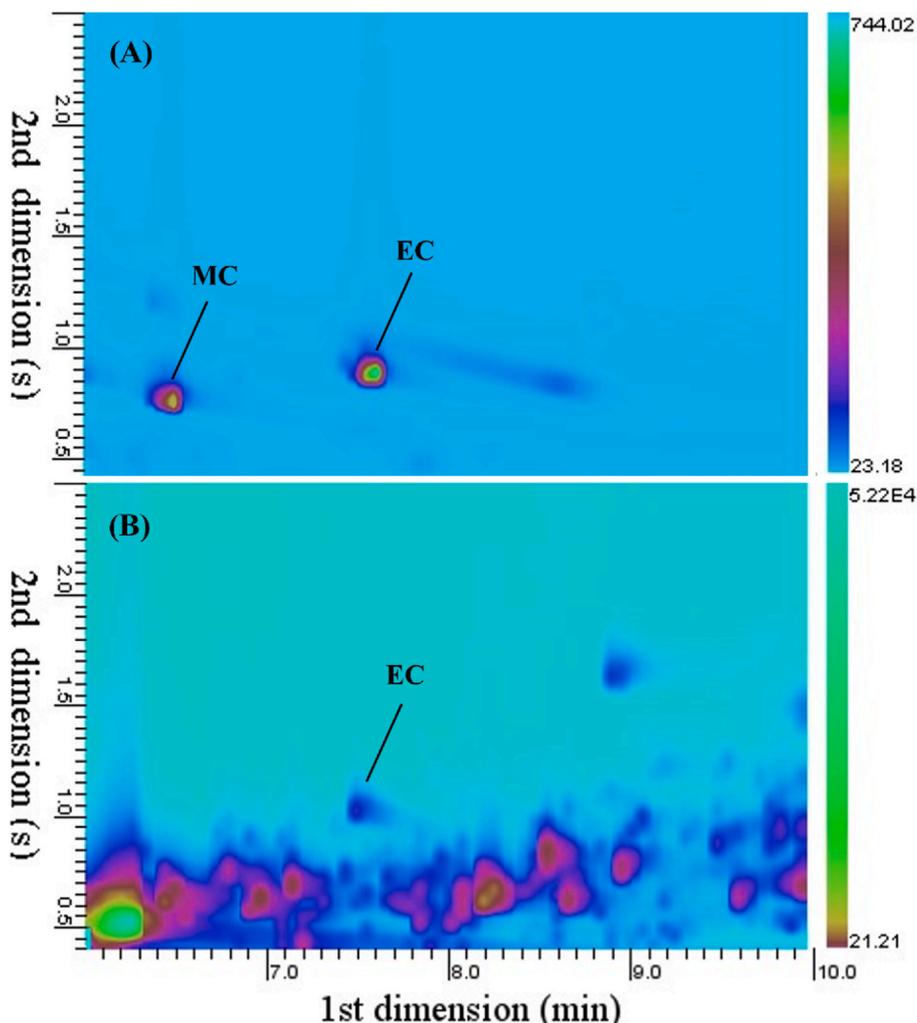


Fig. 3. Color plot of methyl carbamate (MC) and ethyl carbamate (EC). The relative retention time (RT) of EC to MC's RT allowed the identification of the EC signal. A) Direct injection of the MC and EC standard solution (10.00 mg L^{-1}). B) Result after DA-DLLME of cachaça sample spiked with EC ($415.00 \text{ } \mu\text{g L}^{-1}$).

decided to reduce the alcohol content. This adjustment allowed the extraction solvent to disperse effectively. The reduction of the alcohol concentration can be achieved by diluting the sample; therefore, a fine-tuning of the alcohol concentration was necessary. HPLC/RID analysis could provide accurate data (Avila et al., 2018), and factorial design data showed optimal dilution.

The extraction solvent volume had to provide a satisfactory enrichment factor and enable dispersion for phase separation. The best combination of dilution and extraction solvent volume was selected based on the factorial design.

The innovation that allowed the EC extraction came from the observation that adding chloroform, followed by the alcoholic sample, and then by a rapid addition of the phosphate buffer solution as a diluent, turned the mixture immediately turbid, evidence of emulsion formation. Adding a diluent can change the solubility of the extraction solvent and the number of phases in the system. El-Deen et al. (2023) reported that solubility adjustment can be a dispersion technique, although temperature control has been the primary method. For example, cooling a sample containing deep eutectic solvents (DES) reduces the solvent's solubility, forming a cloudy two-phase system (Abdi et al., 2020). The droplets formed by the disturbance of the solution equilibrium, which forms the cloudy mixture, are stable, and the extraction is performed with robust results (Farajzadeh et al., 2018). In the present work, the system changes that reduce the solubility of the extraction solvent is the rapid addition of a diluent. When the extraction

solvent is present in a homogeneous system with the analyte, both can interact without the surface area limitations in a two-phase system. Nanoemulsion formation by the emulsion inversion point (EIP) technique can illustrate this phenomenon. It is a low-energy method (Gupta et al., 2016) where the system undergoes an inversion point during this dilution process, and tiny droplets can be formed. Therefore, dilution with a buffered solution was tested as a novel method for DLLME droplet formation suitable for distilled beverages with high alcohol content.

The choice of a buffered solution as the diluent also became essential for controlling the pH of the extraction method. The pH adjustment favors the neutral form of a Lewis acid or base analyte. It can improve extraction efficiencies, as the neutral analyte form may have a greater affinity for the non-polar extracting solvent (Psillakis, 2019). Liao et al. (2013) optimized EC extraction using DLLME in wine and reported no significant effect on the response in the pH 7.0 to 9.0 range. To ensure that the molecular EC rather than its charged ion predominated in the present study, a phosphate buffer solution ($\text{pH} = 7.2 \pm 0.1$) was used for dilution.

Regarding the salting-out effect referenced in the literature, it was observed that adding NaCl increased the chromatographic signal, as Machado et al. (2013) reported when using solid-phase microextraction (SPME). However, the authors stated this was unsuitable for SPME routine analyses with direct immersion (DI), as fiber conditioning after each extraction is required. For DLLME, the value of 15% (w/v) NaCl resulted in the best responses by GC-MS/MS when analyzing wine

samples (Liao et al. 2013). It was opted to test the effect of NaCl on extraction efficiency.

EC is a polar compound highly soluble in water, and using polar solvents can be adequate for matrix modification-assisted headspace single-drop microextraction (Ma et al., 2022), but they are not suitable for LLE. For the DLLME, the extraction solvent must be insoluble in the matrix (Ahmad et al., 2015), have a high partition coefficient, low volatility, and be compatible with the analytical instrument (Kannouma et al., 2022). The extraction solvent chosen was chloroform, which is reportedly compatible with EC extraction, leading to a robust analytical performance (Liao et al., 2013; Moreno-González et al., 2015).

Combining the possibility of dispersing the extraction solvent using a low-cost diluent in a low-energy method that can lead to a nanoemulsion with simple steps DA-DLLME was developed and optimized for the analysis of EC by gas chromatography.

3.2. Method validation parameters

First, it was checked whether the proposed methodology has a matrix effect. Therefore, analytical graphs for the external and internal calibration (with the matrix addition) were generated in the concentration range from 145.00 to 415.00 $\mu\text{g L}^{-1}$. For the external calibration curve, the linear fit ($R^2 = 0.9703$) was described by the equation $y = 1295.1005 + 20.6804 * x$, and the Cochran test evaluated the homogeneity of variance. For linearity, homoscedasticity was observed ($C_{\text{calc}} = 0.3188 < C_{\text{tab}} = 0.5612$). The internal calibration curve ($R^2 = 0.9731$) was described by the equation $y = 979.9259 + 22.7765 * x$, and the data were homoscedastic ($C_{\text{calc}} = 0.3532 < C_{\text{tab}} = 0.6161$). The F-test ($\alpha = 0.05$) indicated that there is homogeneity in the variance of internal and external calibration curves ($F_{\text{calc}} = 1.657 < F_{\text{tab}} = 2.084$), and the T-test suggested that both inclinations are equivalent ($T_{\text{calc}} = 1.792 < T_{\text{tab}} = 2.024$), therefore it can be assumed that to matrix effect was not observed for this method. Numerical values for the validation parameters evaluated are provided in Table 1.

The LOD and LOQ values were 37.52 $\mu\text{g L}^{-1}$ and 125.07 $\mu\text{g L}^{-1}$, respectively. The recovery results, recommended as performance criteria, should be between 80 and 120% (Schuh & Pozebon, 2021). The DA-DLLME with GCxGC/FID analysis was performed with precision and recovery rate within an adequate range for the chromatographic technique, and the results are summarized in Table 1. This performance is due to the high selectivity given by the orthogonality of the two chromatographic columns of the system, which resulted in an excellent chromatographic resolution of the analyte from the cachaça, as shown in Fig. 3.

Using a flame ionization detector to quantify EC is challenging for detecting small molecules as the signal intensity is significantly dependent on the number of carbons in the molecule structure (Poole, 2021). Only through a considerable enrichment factor of DA-DLLME combined with the high sensitivity provided by the GCxGC system, the FID signal was strong enough to detect small concentrations of EC, a two carbon molecule.

The effectiveness of the chromatographic system used in the present work has already been demonstrated in a previous work by Novaes et al. (2024), who performed the determination of several compounds with low molecular mass and high volatility. Another particularity in the present work was using the differential flow modulator in the GCxGC system, which is a simple and inexpensive modulator compared to the typically used thermal modulators (Seeley, 2021). In this valve-based

Table 1
Validation parameters results for DA-DLLME-GCxGC/FID.

Concentration ($\mu\text{g L}^{-1}$)	Recovery (%)	Intra-day (RSD%)	Inter-day (RSD%)
190.00	104.09	9.98	11.07
280.00	101.94	6.37	7.80
390.00	88.83	7.25	10.05

technique, the flow from the first column is collected in a sample loop and diverted to the second column after actuation. The flow rate of the carrier gas in the second column is higher than in the first, so the time to fill the loop is greater than the drain time. With cyclic modulation periods, the primary peaks are constantly converted into a high-intensity pulse, resulting in a sensitivity about eight times higher than a one-dimensional GC (Bahaghighat et al., 2019). Differential flow modulator technology increased the sensitivity of an FID by detecting sharp pulses instead of a broader area peak. As a result, by performing the DA-DLLME as a sample preparation method and exploring the GCxGC/FID advantages, the EC analytical signal from cachaças samples could be detected and quantified. Therefore, a simple, fast, robust, environmentally friendly, and economical method was developed.

3.3. Determination of real samples

The analytical results for the real samples are summarized in Table 2. The results showed an alcohol content ranging from 39.69% to 49.18%, with some samples showing values slightly different than those reported by the manufacturers. Regarding EC concentration, six samples (A, B, C, G, H, and K) presented concentrations above 415 $\mu\text{g L}^{-1}$, exceeding acceptable limits in many regulations. Sample D showed a level of 145.42 $\mu\text{g L}^{-1}$. Samples E, F, and J had concentrations below the LOQ, suggesting a minimal presence or undetectable levels of EC. Some samples with high alcohol content, such as C, G, and H, showed elevated EC concentrations ($>415.00 \mu\text{g L}^{-1}$). The results are consistent with the carbamate values reported in previous work analyzing cachaças. Caruso et al. (2010) found that 32 samples from 61 cachaças from five Brazilian states ranged in concentration from 151.00 to 960.00 $\mu\text{g L}^{-1}$. Using GC-MS, sugar cane spirits from the Brazilian were analyzed by Zacaroni et al. (2015), and all samples had an EC concentration below 150.00 $\mu\text{g L}^{-1}$. Bortoletto and Alcarde (2015) found that 27.6% of the analyzed samples had EC concentration above 300.00 $\mu\text{g L}^{-1}$.

The literature demonstrates that the sugarcane variety or storage conditions of the manufactured cachaça have a low impact on the EC concentration (Nóbrega et al., 2009; D'Avila et al., 2016). Therefore, the EC concentration above the legal limits found by the DA-DLLME-GCxGC/FID methodology is probably unrelated to these variables. The inadequate removal of the head and tail fractions during the distillation of the fermented must is a critical factor that increases EC concentration. Mendonça et al. (2016) evaluated the EC concentration in cachaças produced with different yeast sources. No correlation could be found between those factors. Because there is no support in the literature, it is not possible to correlate the EC concentrations found above the legal limits in the present work to contamination, poor quality, or lack of microbiological control of the yeast strains.

The determination of EC in cachaça stored in oak, amburana, jatobá, balsa, and peroba, as well as in glass containers, did not yield conclusive results, as the instrumental response for the samples analyzed by Santiago et al., 2017 were below the LOQ. They reported that the EC

Table 2
Results of the analysis of actual cachaça samples and volume of sample and water used for alcohol content correction for a final volume of 10 mL.

Sample	Alcohol content reported (%)	Alcohol content found (%)	EC concentration ($\mu\text{g L}^{-1}$)
A	45	44.10	>415.00
B	44	43.68	>415.00
C	48	49.18	>415.00
D	48	47.65	145.42
E	47	47.46	$< \text{LOQ}$
F	42	44.84	$< \text{LOQ}$
G	47	49.11	>415.00
H	49	48.39	>415.00
J	40	40.15	$< \text{LOQ}$
K	40	39.69	>415.00

concentration increased during storage in wood containers, and time no storage influenced the formation of EC in cachaça stored in glass containers, but with different analyses below the limit of quantification since it was reported that an increment of less than $3 \mu\text{g L}^{-1}$ of EC over a year of barrel storage, it is probable that the concentration above the $210 \mu\text{g L}^{-1}$ mark in the present work is not due to the aging in contact with wood.

The use of copper still (alembic) is often associated with the artisanal production of cachaça. Since it is a batch process that requires the separation of three fractions of the distillate (head, heart, and tail) based on the alcohol content, it was found by Reche et al. (2007) that the removal of the head fraction could help to reduce the EC in the final product. EC is very soluble in ethanol, and the head fraction contains a high alcohol concentration. The authors also noted that the EC and phenylmetanal are the most important substances when clustering samples are continuously produced industrially in stainless steel columns. Baffa Júnior et al. (2011) quantified the copper concentration and its possible correlation with EC concentration at different stages of the sugar cane fermentation process, in different distilled fractions, and in the vinasse of the cachaça production process. It was found that only the hearth fraction of cachaça was below the applicable legal limits for the contaminant, which is consistent with the results of Reche et al. (2007). Confirmatory data were later collected by Andrade Sobrinho et al. (2009), who found lower EC levels in alembic cachaça than in samples produced by column distillation, as it is a continuous process, and the three fractions are not separated. Lower EC levels were found in cachaças from still distilled at low temperatures and high reflux rate. This behavior was found in a study examining the influence of distillation processes on EC in cachaças (Bruno et al., 2007), where the contaminant was measured in samples taken directly from stills. Alcarde et al. (2012a) show that double distillation can reduce the EC concentration in the sugar cane spirit, and later that the EC concentration decreases after a second distillation in the hot head, head cooler, dephlegmator and rectifier pot stills (Alcarde et al., 2012b). The chemical composition of sugar cane spirit obtained by single-distillation, double-distillation, and redistillation was the subject of investigations (Silva et al., 2020). All samples were below the legal limit. The single distillation method resulted in the higher EC concentration observed, and in the redistilled alcohol, the response was below the LOD.

The evidence suggests inadequate head and tail cutting during distillation can produce cachaças with a higher EC content and an inefficient control of the productive process. In cachaças, distillation plays the most important role in the quality of the final product compared to other possible sources of contamination in terms of the sugar cane or yeasts used, storage conditions, and exposure light. The cachaças analyzed in this work were above the legal limit, probably due to poor control of the distillation process rather than external storage factors. Since the samples analyzed in the present work are reportedly from alembics, it is possible to assume, supported by the literature, that only four of the cachaças (D, E, F, J) within the legal limits had their distillation head fraction adequately removed.

3.4. Method comparison

Results reports for using GC-MS without any sample preparation by direct injection of cachaças samples for EC analysis showed that the LOQ is between $10.0 \mu\text{g L}^{-1}$ and $50.0 \mu\text{g L}^{-1}$. The GC-MS method with direct injection can be advantageous as it does not require sample pretreatment. Still, it depends on the sensitivity of the mass detector whether lower values for LOD and LOQ are achieved. The use of GC-MS with a prior evaporation of the samples with a nitrogen stream and dilution of the dried material with methanol before chromatographic analysis has been reported (Caruso et al., 2010). This sample preparation step, LOD, and LOQ of $5.00 \mu\text{g L}^{-1}$ and $20.00 \mu\text{g L}^{-1}$, respectively, could be achieved, proving that this sample preparation method could be more efficient than direct injection. However, it requires more steps and

reagents.

The first report on solid-phase microextraction (SPME) for the determination of EC by GC-MS was presented by Machado et al. (2012). The evaluation of extraction mode, sample pH, temperature, and extraction time, followed by a kinetic study, showed that the optimal parameters for PA fiber were direct extraction in samples with 10% of ethanol buffered at pH 7.0, requiring an extraction time of 20.00 min at 50.0°C stirring at 900 rpm. The method resulted in the lowest LOQ for chromatography, the expense of a long extraction time, and the use of fragile SPME fibers.

In a later work using a similar method previously described by Anjos et al. (2011), EC was quantified in cachaças from copper distilleries by HPLC-FLD synthesizing N-xanthylethylcarbamate (CNXE) from EC and a 9-xanthinol (Machado et al., 2013). The validated method was suitable for liquid chromatography. It was characterized by a reproducible sample preparation that achieved lower precision values reported for EC determination with a sample preparation time of 61.0 min.

The methodology of QuEChERS (Guerreiro et al., 2018) by the salting-out of ethanol in cachaça through the use of potassium carbonate allowed the authors to extract the ethanol and EC from the sample. With minimal sample preparation, they could perform GC-MS analysis on the order of micrograms per liter, as reported. Without a prior alcohol content determination, this method could introduce a high degree of uncertainty as it relies on constant partition values for both EC and ethanol after salting out. In the last published works on this topic, no further advances or improvements in EC determination by a chromatographic methodology in cachaça were reported (Silva et al., 2020; Vilela et al., 2021).

The DA-DLLME before GCxGC/FID analysis was performed with a precision and recovery rate that was concise in the literature without the expense of long extraction times or high volume of reagents and solvents. Besides, it was possible to overlap the FID limitations for small molecules detection, and its signal for the EC was intense enough to reach values of $37.50 \mu\text{g L}^{-1}$ for the contaminant detection.

3.5. Analytical greenness

In addition to analytical performance, the new method was evaluated using a sustainable sample preparation metric developed by González-Martín et al. (2023) to assess green parameters. The proposed metric follows 10 principles of Green Sample Preparation (GSP) and focuses solely on the sample preparation method. Evaluations such as T, the Green Analytical Procedure Index (GAPI), the Analytical GREENness (AGREE), and the ComplexGAPI, HEXAGON, and RGB do not allow an efficient comparison of the extraction procedure since the instrumentation parameters are also evaluated (González-Martín et al., 2023). Therefore, the greenness of DA-DALLME was calculated using an interactive datasheet provided by the authors (González-Martín et al., 2023). It scored 6.53 out of 10.00, and the pictogram of the sample preparation metric for sustainability for DA-DLLME is available as supplementary material.

For the proposed metric, sample volumes of 10.00 mL or less, extraction times of 5.0 min or less, and the absence of additional post-extraction steps contribute to greater environmental friendliness. Additionally, the lack of energy-consuming devices for dispersion and the absence of temperature control further enhances the eco-friendliness of the sample preparation process. Using chloroform, a chlorinated solvent, was the most critical aspect that lowered the score, represented by the red square, also present in the conventional DLLME. In the overall evaluation, the DA-DLLME was superior to traditional DLLME with a score of 6.21 (González-Martín et al., 2023). The fast and low-energy extraction and the minimal steps to perform the sample preparation are the more significant advantages of DA-DLLME.

4. Conclusion

In this study, a novel dispersion method for DLLME was developed, optimized, and validated for the analysis of EC by GCxGC/FID, which showed promising results in terms of environmental sustainability. The method provided a quick and straightforward sample preparation step, showing a linear response and suitability for the intended target. However, using chloroform as an extraction solvent remains a concern due to its limited environmental friendliness. Eliminating NaCl and minimizing the amount of extraction solvent added to the system positively impacted the analytical response. The method made it possible to determine the EC in various cachaças in a robust, repeatable, and precise manner. In addition, excellent analytical performance was achieved within the limits of current legislation for this contaminant. Further studies should be conducted to check the suitability of the method for analyzing other contaminants in cachaças and other distilled beverages, such as brandy and whiskey, and to verify the extraction performance of chromatograph detectors with higher detectability.

CRedit authorship contribution statement

Paulo Salles Neto: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Jhonatan Bispo de Oliveira:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Flávia Barbosa Magalhães Alvarenga:** Methodology, Investigation, Data curation. **Carlos Augusto Rosa:** Writing – review & editing, Formal analysis, Conceptualization. **Patrícia Santiago de Oliveira Patrício:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Conceptualization. **Patterson Patrício de Souza:** Writing – review & editing, Writing – original draft, Validation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2025.111187>.

Data availability

Data will be made available on request.

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