

# Coupling of a streamlined solid-liquid extraction protocol with LC-ESI-MS/MS for the regular oversight of illegal bromate additive use: An accurate and sensitive determination method in preliminary and bakery products

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## ABSTRACT

A reliable solid-liquid extraction protocol coupled with liquid chromatography-electrospray ionization-tandem mass spectrometry in the negative-ion mode was developed and validated for illegal bromate determination in preliminary and bakery products. Crude and dried-treated samples were directly extracted with acetonitrile-water (4:1, v/v). Bromate was determined using a Phenomenex Synergi™ Polar reversed-phase column and MS/MS under multiple reaction monitoring. The chosen solvent efficiently extracted bromate with all applied extraction-assisting techniques ( $p > 0.05$ ). Although this assay avoids cleanup procedures, matrix effect of  $< -11\%$  was achieved. Rapid bromate separation in only 8 min was attained by a reversed-phase column. In both commodities, linearity range,  $R^2$ , recovery%, repeatability, intermediate precision, LOD and LOQ results were 0.05–100 ng mL<sup>-1</sup>,  $> 0.9999$ , 88.6–103%, 2.93–9.80% and 9.64–10.10%, 0.015 µg kg<sup>-1</sup> and 0.05 µg kg<sup>-1</sup>, respectively. Out of 288 tested real samples, 13.9% of violations were observed. This high-sensitivity protocol offers effective oversight and consumer protection.

## 1. Introduction

Egypt is one of the world's largest consumers of wheat flour, mainly for making Baladi bread and other bakery products (FAS/USDA-Global Market Analysis, 2023; McGill, Prikhodko, Sterk, & Talks, 2015). Bakery products typically include flour, yeast, water, salt, and sometimes bread improvers like potassium bromate (KBrO<sub>3</sub>) (Abu-Obaid, AbuHasan, & Shraydeh, 2016). Even though KBrO<sub>3</sub> is a possible human carcinogen (Group 2B), causes several health complications ("IARC," 1999), and is globally and nationally regulated as a banned substance (European Parliament and the Council of the European Union, 2008; NFSA. National Food Safety Authority, 2020), it is still illegally used (Abu-Obaid et al., 2016). On the other hand, and under conservative circumstances, a maximum of 0.0075 wt% is permissible for flour treatments with KBrO<sub>3</sub> (USFDA, 2023). This is because technological purposes are the major stimulus toward the treatment of flour and other preliminary products with KBrO<sub>3</sub>, as it is affordable and effective in im-

proving low-quality gluten (Shanmugavel et al., 2020). This calls for efficient analytical methods capable of providing accurate and sensitive bromate (BrO<sub>3</sub><sup>-</sup>) determinations in various bakery products.

Previous literature have described several techniques for BrO<sub>3</sub><sup>-</sup> determination, including ion chromatography (IC) with different detection techniques like conductivity detection (IC-CD) (Y. Shi, Liang, Cai, & Mou, 2006), tandem mass spectrometry (IC-MS/MS) (Aggrawal & Rohrer, 2020), and inductively coupled plasma mass spectrometry (IC-ICP/MS) (H. Shi & Adams, 2009). Additionally, protocols utilizing high-performance liquid chromatography (HPLC) with post-column derivatization followed by UV detection (Yokota et al., 2012) and LC-MS/MS have been reported for BrO<sub>3</sub><sup>-</sup> analysis in various commodities (Anastassiades et al., 2021; Dong, Xiao, Xian, Wu, & Zhu, 2019; Xian et al., 2017). However, several limitations have been identified in the existing methods for BrO<sub>3</sub><sup>-</sup> detection in various commodities. While both ion chromatography post-column reaction and HPLC post-column derivatization offer high sensitivity and selectivity, they require sophisti-

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cated instrument setup, multi-step sample pretreatments, and potentially toxic derivatization chemicals (Delcomyn, Weinberg, & Singer, 2001; Yokota et al., 2012). Although conductivity detection methods have provided sensitive determinations, a limited selectivity has been reported, especially when applied to complex food commodities containing multiple ionic chemicals (Aggrawal & Rohrer, 2020). Though IC-ICP/MS boasts low detection limits and minimal interference, its high cost, complexity, and multi-step sample cleanup hinder its widespread use (Michalski & Lyko, 2013). Therefore, recent studies have increasingly turned to LC-MS/MS as the gold standard for  $\text{BrO}_3^-$  analysis. Despite its high cost, LC-MS/MS offers exceptional selectivity and sensitivity, making it ideal for the reliable and accurate determination of  $\text{BrO}_3^-$  residues in complex food matrixes (Dong et al., 2019). While  $\text{BrO}_3^-$  is a polar anion with a small molecular weight and is typically separated by normal-phase columns (Xian et al., 2017), separations using reversed-phase columns have also been reported (Dong et al., 2019); nevertheless, further investigations are needed.

Sample preparation, on the other hand, is a key factor in efficient  $\text{BrO}_3^-$  extraction from complex commodities. Various studies have demonstrated the versatility of sample cleanup procedures such as dispersive solid phase extraction (d-SPE) employed in QuEChERS-based protocols for removing interfering substances, ensuring accurate analysis by LC-MS/MS in flour (Xian et al., 2017) and fresh fruits and vegetables (Dong et al., 2019). Although QuEChERS is a cost-effective method with high recoveries, it often requires additional steps and certain modifications, particularly with commodities of variable and complex composition, to reach the best sensitivity (Rodríguez-Carrasco et al., 2018). Notably, the reported limit of quantitation (LOQ) values by (Xian et al., 2017) and (Dong et al., 2019) were  $6 \mu\text{g kg}^{-1}$ , and  $15 \mu\text{g kg}^{-1}$ , respectively. Interestingly, the solid-liquid extraction (SLE) protocol is a typically preferred approach owing to its simplicity and effectiveness (Castilla-Fernández, Rocío-Bautista, Moreno-González, García-Reyes, & Molina-Díaz, 2022). For instance, a method known as QuPPE-PO-Method has been reported by (Anastassiades et al., 2021) to undergo SLE for  $\text{BrO}_3^-$  and highly polar pesticide residues from fresh fruits and vegetables, followed by LC-MS/MS measurement, with an achieved LOQ value of  $20 \mu\text{g kg}^{-1}$ . Given these advantages, it is beneficial to perform a thorough investigation into the wide applicability of the SLE protocol to test ultra-low levels of  $\text{BrO}_3^-$  residues in complex foods like flour and bakery products. Hence, a careful experimental design of a sample processing protocol for  $\text{BrO}_3^-$  additive analysis is crucial. Univariate statistical evaluations, like analysis of variance (ANOVA) and descriptive statistics, are fundamentally applied to compare different sample preparation procedures. These comprehensive comparisons help ensure effective method development and optimization by revealing interactions between processing variables (Percival, Gibson, Leenders, Wilson, & Grootveld, 2021). This would allow for effective, regular, and reliable oversight, ensuring consumer safety.

In the current study, it is hypothesized that univariate statistics will differentiate between SLE steps and extraction-assisting techniques for efficient and accurate LC-ESI-MS/MS  $\text{BrO}_3^-$  determination. Therefore, this study aims to develop and validate a statistically optimized SLE protocol combined with LC-ESI-MS/MS for the reliable and efficient analysis of illegal  $\text{BrO}_3^-$  additive use in preliminary and bakery products. Full validation of the optimized method, adhering to the EU requirements outlined in the Eurachem 2014 guideline (Magnusson & Örnemark, 2014), will be conducted on flour and Baladi bread samples. Subsequently, the validated method will be applied to 288 real samples from the Egyptian market to identify potential violations of national and international regulations concerning  $\text{BrO}_3^-$  misuse, enhance local market oversight to ensure food safety standards, and maximize consumer protection by minimizing exposure to potentially harmful  $\text{BrO}_3^-$  additives.

## 2. Materials and method

### 2.1. Chemicals and standard solutions

Ultrapure water was produced by the Milli-Q UF-Plus purification system, with a resistivity  $>18.0 \text{ M}\Omega \times \text{cm}$  and a total organic carbon (TOC)  $<5 \text{ ppb}$  (Millipore, Darmstadt, Germany). Methanol (MeOH) of LC-MS grade with a purity  $\geq 99.99\%$  was purchased from Supelco® (Darmstadt, Germany). Acetonitrile (MeCN) of HPLC grade (99.9%) was purchased from Carlo Erba (Val-de-Reuil, France). Formic acid (FA) of HPLC grade with a purity  $\geq 99\%$  was purchased from Carlo Erba (Val-de-Reuil, France). An ACS reagent of  $\text{KBrO}_3$  with a purity  $>99.8\%$  was purchased from Sigma-Aldrich (St. Louis, USA). A bromate stock standard solution of  $1000 \text{ mg L}^{-1}$  was prepared in a 10 mL volumetric flask by dissolving  $0.0132 \text{ g}$  of  $\text{KBrO}_3$  in ultrapure water. Two working standard solutions were respectively prepared at concentration levels of 100 and  $1000 \text{ ng mL}^{-1}$  by subsequent dilution of the stock standard solution with an appropriate volume of MeCN-water mixture (7:3, v/v) in a 10 mL volumetric flask. The working standard solutions are used to spike samples for quality control testing and to create a set of matrix-matched calibration (MMC) levels at concentrations ranging from  $0.05 \text{ ng mL}^{-1}$  to  $100 \text{ ng mL}^{-1}$ . All standard solutions were stored in a refrigerator at  $4 \pm 2 \text{ }^\circ\text{C}$ . Before proceeding with routine work analysis, working standard solutions were maintained at the ambient temperature ( $23 \pm 2 \text{ }^\circ\text{C}$ ).

### 2.2. Instrumentation and analysis conditions

A system of an Exion LC™ coupled with a Sciex QTrap 6500+ tandem mass spectrometer was purchased from (Applied BioSystems/Sciex, Toronto, Canada). Under several analysis conditions, separation efficiency was tested on various LC columns, including the Kinetex XB-C18  $100 \text{ \AA}$  column ( $150 \text{ mm} \times 4.6 \text{ mm}$ ,  $3.5 \mu\text{m}$ ) (Phenomenex, Utrecht, Netherlands), Thermo Scientific™ Hypercarb™ Porous Graphitic Carbon LC Column ( $100 \text{ mm} \times 2.1 \text{ mm}$ ,  $5 \mu\text{m}$ ) (Thermo Fisher Scientific, Waltham, MA, USA.), Acclaim™ RSLC Polar Advantage II  $120 \text{ \AA}$  column ( $75 \text{ mm} \times 3 \text{ mm}$ ,  $3 \mu\text{m}$ ) (Thermo Scientific, Asheville, NC, USA), and Phenomenex Synergi™ Polar-RP  $80 \text{ \AA}$  LC column ( $250 \text{ mm} \times 4.6 \text{ mm}$ ,  $4 \mu\text{m}$ ). Finally, the chromatographic separations were performed on the later column that was purchased from (Phenomenex, California, USA), and maintained in the column compartment at a temperature of  $40 \pm 2 \text{ }^\circ\text{C}$ . The sample tray was kept at a cool temperature of  $5 \text{ }^\circ\text{C}$ , and the applied injection volume was  $5 \mu\text{L}$ . At a flow rate of  $0.4 \text{ mL min}^{-1}$ , a multi-step gradient elution program was carried out using a mobile phase composition reported by (Dong et al., 2019) of 10-mM ammonium acetate in water with MeCN (9:1, v/v) ( $\text{pH } 5.5 \pm 0.05$ ) in reservoir (A) and MeCN in reservoir (B) over a run time of 8 min. The employed gradient was as follows: start at 0% B for 1.5 min, linearly increase to 10% B till 3.5 min, remain in this condition till 6.95 min, and finally return to the initial conditions in 0.05 min, holding another 1.0 min for equilibration, with an overall run time of 8 min. A unit mass resolution was specified for Q1 and Q3. The MS/MS operational parameters were set as follows: the ion source was a Turbo Spray Ion Drive with an Ion Spray voltage of  $-4500 \text{ V}$  and a temperature of  $450 \text{ }^\circ\text{C}$ . The medium collision gas was nitrogen, with a purity of 99.999%. The pressure of the curtain gas was  $137.8 \text{ kPa}$  while the pressure of ion source gases 1 and 2 was  $344.5 \text{ kPa}$  and  $413.4 \text{ kPa}$ , respectively. At declustering potentials of  $-75$  and  $-35 \text{ V}$  and a collision energy of  $30 \text{ eV}$  each, two emergent transition ions from a parent ion of  $126.7 \text{ [M-H]}^-$  were used for quantitation and further confirmation under multiple reaction monitoring (MRM) with negative ion modes. The first transition ion was  $126.7 \rightarrow 110.8 \text{ m/z}$  (quantifier ion), while the other one was  $126.7 \rightarrow 94.7 \text{ m/z}$  (qualifier ion). Further identity confirmation was implemented using the  $^{81}\text{BrO}_3^-$  emerged transition ions ( $128.7 \rightarrow 112.8 \text{ m/z}$  and  $128.7 \rightarrow 96.7 \text{ m/z}$ ). According to EU

guidelines for confirmatory methods (European Commission, 2021), the signal-to-noise ratio (S/N) of both ionic transitions must be  $>3$ , and the analyte's ion ratio must be within  $\pm 40\%$  relative deviation of that of the MMC at comparable concentrations measured under identical conditions. The  $\text{BrO}_3^-$  has a qualifier-to-quantifier ion ratio of 26%. Analyst® software 1.7.2 (Applied BioSystems/Sciex, Toronto, Canada) was used for instrument control, method acquisition, data processing, and interpretation.

### 2.3. Equipment and apparatuses

A Binder D 240 drying and heating chamber (Tuttlingen, Germany) for sample drying. This chamber offers forced convection, adjustable temperature (up to  $300\text{ }^\circ\text{C}$ ), and programmable incubation times (up to 99.59 h). Dried samples were then crushed till fine powder is obtained using a Waring 8010S-HGBTWTS3 blender (Torrington, USA) with a 1-l stainless steel container and a timer function of up to 180 s. To determine the moisture content of the samples, a Mettler-Toledo HR73 halogen moisture analyzer (Greifensee, Switzerland) with a standard drying program ( $105\text{ }^\circ\text{C}$ ) was used.

Extraction-assisting techniques employed were mechanical shaker, ultrasonic, water bath with mechanical agitation. A mechanical shaker of a Spex™ sample prep 2010 Geno/Grinder™ was obtained from Thomas Scientific (Metuchen, USA) for automated homogenization and cell lysis of various plant and animal tissues. This high-throughput system offered adjustable speeds ranging from 500 to 1750 strokes per minute. A BSH shaking thermostatic bath obtained from Raypa (Barcelona, Spain) to facilitate extractions. This versatile water bath provided adjustable temperature control from room temperature to  $100\text{ }^\circ\text{C}$ , shaking speeds between 10 and 150 rpm, and programmable incubation times up to 99.5 h. An Elmasonic S 60 (H) ultrasonic cleaning unit (Singen, Germany) was incorporated for sample cleaning. This unit offered high-performance 37 kHz transducers, a control knob for setting continuous or pulsed operation from 1 to 30 min, and temperature-controlled ultrasonic operation ranging from 30 to  $80\text{ }^\circ\text{C}$  (available only in heated models). For sample purification, a cooling centrifuge of a model Z 446 K with a relative centrifugal force (rcf) of 16,020 g for  $10 \times 50\text{ mL}$  has been obtained from HERMLE Labortechnik GmbH

(Wehingen, Germany). A 4042–6" Test Sieve, 0.177 mm particle size, Half Height, Stainless Frame - Stainless Cloth has been obtained from HOGENTOGLER & CO. INC. (COLUMBIA, USA).

### 2.4. Sampling and sample preparation

A total number of 288 samples were purchased from the domestic market in Egypt to test the  $\text{BrO}_3^-$  contents. Out of the collected commodities, 26 of samples tested were preliminary products while the remaining samples (262) were bakery products. The preliminary samples included products of 22 flour, 3 baking powder, and 1 cake powder. All other bakery products were 38 Baladi bread, 38 soft baguettes, 39 cake, 11 croissants, 18 bread rolls, 11 pizza, 28 pate, 9 toast, 14 noodles, 28 biscuits, 17 flour wraps, 1 pizza crust, 8 burger bun, and 2 bread stick (Table S1).

Samples of Baladi bread and all other bakery products were sliced and dried as per (Y. Shi et al., 2006). Briefly, sliced baked samples were dried overnight at  $50\text{ }^\circ\text{C}$  in an oven, then crushed using a mortar and pestle and further homogenized with a blender to a 0.177 mm powder. To ensure all dried samples were suitable for  $\text{BrO}_3^-$  analysis with minimal weight-related errors, their moisture contents were further measured (not  $>5\%$ ). Finally, samples were frozen in well-sealed plastic bags to prevent deterioration and compositional changes. Before analysis, samples were thawed to room temperature, thoroughly mixed to ensure homogeneity, and dried again for 1 h (Fig. 1).

### 2.5. Solid-liquid extraction procedure

A revisited and entirely optimized QuPPE-PO-Method (Anastassiades et al., 2021) was applied for the solid-liquid extraction of  $\text{BrO}_3^-$  from preliminary and bakery products. In this respect, an aliquot of  $5 \pm 0.02\text{ g}$  of homogenized sample was weighed into a 50 mL polypropylene falcon tube. For quality control testing, an appropriate volume of the  $\text{BrO}_3^-$  working standard solution was added to the blank samples at a concentration level of  $1\text{ ng g}^{-1}$  to test the recovery percentage. Spike samples were allowed to stand at room temperature for 5 min to facilitate the interaction with the matrix components. Afterward, 20 mL of MeCN-water (4:1, v/v) was added to each tested sam-

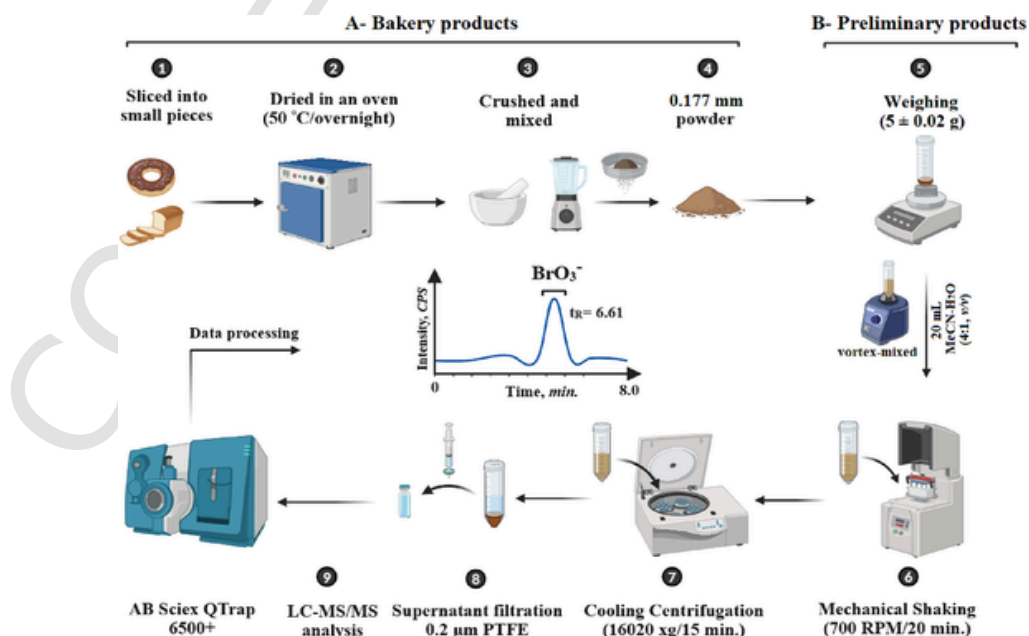


Fig. 1. A schematic diagram depicts the sample preparation process for a) - bakery products, and b) - preliminary products as part of the proposed protocol for  $\text{BrO}_3^-$  determination.

ple. All samples were vortex-mixed for 1 min until complete homogeneity was attained. Samples were vigorously shaken vertically using a mechanical shaker at 700 rpm for 20 min. A complete phase-out separation was carried out by centrifugation for 15 min under cooling conditions of  $5 \pm 3$  °C at an rcf of  $16,020 \times g$ . The obtained supernatants were then filtered through a  $0.2 \mu\text{m}$  polytetrafluoroethylene (PTFE) syringe filter and placed into a glass vial prior to direct injection into an LC-ESI-MS/MS system. Fig. 1 exhibits a schematic representation of sample processing procedures for  $\text{BrO}_3^-$  analysis in both preliminary and bakery products.

## 2.6. Statistical analysis

All tested extraction protocols at the proposed mixing ratios of an extraction mixture composed of MeCN-water (e.g., 1:1, 3:2, 7:3, 4:1, and 9:1, v/v) were compared in terms of the obtained recovery percentages and statistically assessed using one-way analysis of variance (ANOVA). This test was carried out in order to investigate whether differences obtained in the average recoveries obtained by the employed extraction-assisting techniques (5 replicates, each) are significant or non-significant results. Prior to proceeding with ANOVA testing through the entire method optimization, the normality of the data was initially confirmed by the Shapiro-Wilk test ( $p$  value  $> 0.05$ ), particularly when a small sample size of  $< 50$  is tested. Data proved normally distributed are eligible to perform ANOVA testing to show differences. The applied extraction-assisting techniques included SLE with a mechanical shaker and heat-assisted or induced extraction procedures such as a heated water bath with a mechanical shaker and ultrasonic-assisted extraction. The ANOVA results obtained were considered to have a significant difference when the  $p$  value was  $< 0.05$  and a non-significant difference when the  $p$  value was  $> 0.05$ .

In order to identify the most appropriate extraction mixture composition for efficient  $\text{BrO}_3^-$  extraction from the studied commodities following an application for several extraction techniques, a further statistical analysis was also carried out using descriptive statistics. Mean, median, standard error (S.E.), and confidence level at 95% are the key resulting parameters from this test. Higher mean recovery percentages associated with the lowest possible S.E. were considered the most convenient extraction mixture for successful extraction of  $\text{BrO}_3^-$  residues from preliminary and bakery products.

Further confirmation of the appropriateness of the selected extraction mixture composition for  $\text{BrO}_3^-$  efficient extraction from such complicated commodities in combination with the applied extraction techniques was also implemented using one-way ANOVA testing followed by descriptive statistics. Whereas, the recovery percentages obtained from the analyzed 5 replicates of the selected extraction mixture by the tested extraction techniques were compared and statistically evaluated.

## 2.7. Calibration, matrix effect and quantitation

A multi-level calibration curve was constructed over a concentration range of 0.05 to  $100 \text{ ng mL}^{-1}$  via further dilution of the working standard solutions ( $100$  and  $1000 \text{ ng mL}^{-1}$ ) in MeCN-water (7:3, v/v) to prepare a solvent-based calibration (SBC). Similarly, a set of six points matrix-matched calibration curve (MMC) of 0.05, 0.5, 5.0, 10.0, 50.0, and  $100.0 \text{ ng mL}^{-1}$  was also prepared. This was carried out via spiking the extracted blank matrixes following the proposed assay protocol described earlier with appropriate volumes from working standard solution. Both calibration curves were analyzed as described, where the obtained responses were plotted versus concentration ( $\text{ng mL}^{-1}$ ) and the linear regression coefficient ( $R^2$ ) was recorded per each constructed calibration curve under linear regression equation model. Slopes obtained from both curves were compared and the resultant slope difference % was used to estimate the matrix effect (ME) magnitude as demonstrated in the below equation (eq. 1). ME is deemed tolerable when the ME%

is  $\leq |20|$  %, while it is considered intermediate, and strong signal suppression or enhancements at effects  $> \pm 20 \leq \pm 50\%$ , and  $> \pm 50\%$ , respectively (Marzouk, Shendy, Aboelhassan, Gomaa, & El-Shahat, 2023; Shendy et al., 2019).

To quantify all samples, including real and quality control ones, six-level MMCs were used. The obtained concentration ( $\text{ng g}^{-1}$ ) was calculated using two-level calibrations within a dynamic range that encompassed the expected sample concentration. For quality control testing, recovery was determined as the percentage of the known added  $\text{BrO}_3^-$  concentration ( $\text{ng g}^{-1}$ ) that was recovered from the calculated concentration of the extract using interpolation.

$$\text{ME \%} = \frac{[\text{Slope MMC} - \text{Slope SBC}]}{\text{Slope SBC}} \times 100 \quad (1)$$

## 2.8. Method validation

An in-house validation with a model dependent was implemented in accordance with the Eurachem 2014 guideline (Magnusson & Örnemark, 2014), where trueness expressed as recovery percentage  $\pm$  standard deviation (SD), precision expressed as coefficient of variation percentage (CV%) obtained from both repeatability and intermediate precision tests, limit of detection (LOD), LOQ, linearity range, and selectivity were tested.

Blank samples were spiked at four levels, 0.2, 1, 2, and  $10 \mu\text{g kg}^{-1}$  where a set of 10 replicates per each tested level was analyzed to measure trueness. Similarly, CV% was calculated from the obtained results from repeatability testing at the same levels, meanwhile the CV% of the intermediate precision was measured at the lowest spiking level of  $0.2 \mu\text{g kg}^{-1}$  on three successive day ( $n = 30$ ). Selectivity testing was performed via investigating the elution region of the targeted analyte by comparing blank samples to spiked samples at the LOQ level for identifying any possible interferences that may lead to misidentification and/or in accurate quantitation results. Practical LOD and LOQ values were calculated through further dilution of the studied compound. LOD is the concentration when the peak response signal-to-noise ratio (S/N) is  $\geq 3$ , while LOQ is the concentration when the S/N ratio is  $\geq 10$  times. Assay ruggedness was continuously verified via its routine application to real samples of preliminary and bakery products.

Further confirmation of trueness outcomes was performed using two proficiency testing (PT) samples supplied by the Food Analysis Performance Assessment Scheme (FAPAS) for  $\text{BrO}_3^-$  analysis in water. This is because of unavailability of PT samples in the targeted commodities studied from various PT providers. PT results were assessed on the basis of the obtained z-score value. Where z-score values  $< |2|$  are considered satisfactory. For measurement uncertainty (MU), the directions of Eurachem CITAC Guide CG 4 were implemented (Ellison & Williams, 2012). The validated method practicality were tested by analyzing 288 real samples from the domestic markets and the results evaluated according to EU regulations (European Parliament and the Council of the European Union, 2008) and national authorities (NFSA. National Food Safety Authority, 2020).

## 2.9. Experimental design for method optimization

### 2.9.1. LC-MS/MS optimization

In the current study, experiments were carefully designed to optimize the selectivity and sensitivity of the test method. Among the tested factors were the solvent types of the standard material, column characteristics, mobile phase composition, flow rates, and injection volumes.

### 2.9.2. Sample processing optimization

Major influencing variables on the  $\text{BrO}_3^-$  extraction efficiency were tested. Hence, experiments were designed to optimize the sample size and dilution magnitude, extraction solvent composition, direct contact between the extraction solvent and the tested sample, and the sample's

cleanliness. Notably, in all experiments conducted, spike samples at a concentration level of  $2 \mu\text{g kg}^{-1}$  were analyzed in triplicate. Recovery percentages were selected as the studied response in the conducted experiments. For the applicability demonstration of the optimized SLE protocols, samples detected positive for  $\text{BrO}_3^-$  were subsequently compared. As described earlier, univariate statistical analyses using one-way ANOVA and descriptive statistics were further applied to select optimal sample processing procedures.

### 3. Results and discussion

#### 3.1. MS/MS optimization

In order to make an MS/MS optimization for the studied compound's operational parameters, a  $\text{BrO}_3^-$  standard solution of  $500 \text{ ng mL}^{-1}$  dissolved in ultrapure water was directly infused at a flow rate of  $10 \mu\text{L min}^{-1}$ , and the obtained results were recorded using ESI in negative-ion mode. Automatic scanning was carried out to monitor the most intense ions emerging from parent ions of  $126.7 m/z$  and  $128.7 m/z$ , corresponding to  $^{79}\text{BrO}_3^-$  and its isotope  $^{81}\text{BrO}_3^-$ , respectively. In this process all resultant collision energies (CEs), entrance and exit potentials, and other associated parameters were recorded. Following established protocols (Dong et al., 2019), two transition ions demonstrated both high intensity and stable ion ratios, meeting preset acceptance criteria. The fixed isotope ratios of  $\text{BrO}_3^-$  generate a unique fingerprint in the MS spectrum (Fig. S1). This allows for emphasized selectivity confirmation through the transitions originating from  $^{81}\text{BrO}_3^-$  ( $128.7 \rightarrow 112.8 m/z$  and  $128.7 \rightarrow 96.7 m/z$ ). This has been also confirmed through fine optimization using spike samples of an appropriate concentration. The obtained results confirmed the suitability of  $126.7 \rightarrow 110.8 m/z$  for quantitation purposes while a transition of  $126.7 \rightarrow 94.7 m/z$  was selected as a qualifier ion.

The optimal operational conditions for the initial transition were a dwell time of 100 ms, CE of 30 eV, and a de-clustering potential and entrance and exit potentials of  $-75 \text{ V}$ ,  $-10 \text{ V}$ , and  $-11 \text{ V}$ , respectively. While for the qualifier's operational parameters were a dwell time of 100 ms, CE of 30 eV, and a de-clustering potential and entrance and exit potentials of  $-35 \text{ V}$ ,  $-10 \text{ V}$ , and  $-11 \text{ V}$ , respectively. In accordance with EU guidelines for confirmatory methods (European Commission, 2021), and in order to avoid false-positive results, an established acceptance criteria was considered for correct identification and accurate quantification of the studied compound. This criteria include, chromatographic retention time stability; matching of the retention time of the standard solutions and studied analyte in spiked samples; the presence of the relevant transitions from the analyte molecular peak; ionic transition  $S/N > 3$ ; and ion ratio stability between the quantifier and qualifier peak. Hence, four identification points were obtained and successfully employed in the MRM method: one for the precursor ion and 1.5 for each product ion.

#### 3.2. Bromate standard solubility and sensitivity optimization

The influence of solvent composition on the sensitivity of the studied analyte was carefully investigated. A  $\text{BrO}_3^-$  standard solution was prepared in water and in water-MeCN mixtures with varying ratios (1:1, 2:3, 3:7, 1:4, and 1:9 v/v). The results demonstrated a gradual increase in sensitivity with increasing MeCN content, reaching a maximum at a water-MeCN ratio of 3:7 (v/v). Further addition of MeCN resulted in a decrease in  $\text{BrO}_3^-$  sensitivity. This effect is attributed to the optimized MeCN ratio's ability to reduce the overall viscosity and surface tension of the droplets introduced during ESI, leading to improved dispersion and evaporation. This, in turn, enhances the desolvation of analyte ions and improves proton mobility for the analyte rather than the co-analyte. Contrary to previous protocols (Dong et al., 2019; Xian et al., 2017) and (Anastassiades et al., 2021), which respectively re-

ported a solvent mixture composed of MeCN-water 1:1 v/v and MeOH-water 1:1 v/v as optimal for  $\text{BrO}_3^-$  standard outreach intensities, our proposed assay protocol enables sensitive determination of  $\text{BrO}_3^-$  in preliminary and bakery products. Substantially lower LODs and LOQs were achieved compared to previously reported protocols for  $\text{BrO}_3^-$  analysis in various commodities as will be described later (Table S2).

#### 3.3. LC optimization

Due to the polar nature of the target compound and the complex composition of the tested samples, it is crucial to achieve adequate retention, optimal peak characteristics, and the utmost sensitivity. Therefore, various columns from different suppliers were evaluated using a  $2 \mu\text{g kg}^{-1}$  spike sample under a multi-step gradient elution programs employing a mobile phase consisted of 10-mM ammonium acetate in a water solution with MeCN (9:1, v/v) (pH  $5.5 \pm 0.05$ ) and MeCN. The primary selection criteria for the column chosen for  $\text{BrO}_3^-$  separation were the stationary phase composition, particle size, and column dimensions. Hence, a progressive increase in the initial organic solvent ratio from 10% to 50% was examined within the constructed gradient elution programs in combination with various flow rates of 0.3, 0.4, and  $0.5 \text{ mL min}^{-1}$ . It was found that, the best possible separation performance for each studied column was achieved using a gradient composition with a low initial ratio of 10% organic solvent (MeCN) at a flow rate  $0.4 \text{ mL min}^{-1}$ . While, increasing the starting ratio of organic solvent either caused the targeted analyte to elute rapidly or display poor peak characteristics.

As shown in Fig. 2, the studied compound exhibited sufficient retention on the Kinetex XB-C18 column with a  $t_R$  of 5.56 min, but with limited sensitivity. Consistent with (Dong et al., 2019), a comparable  $t_R$  of 4.66 min was achieved using a diamonsil C18 (2) column ( $250 \text{ mm} \times 4.6 \text{ mm}$ ,  $5 \mu\text{m}$ ) (Table S2). Conversely, the Acclaim<sup>TM</sup> RSLC Polar Advantage II  $120 \text{ \AA}$  column exhibited poor  $\text{BrO}_3^-$  retention ( $t_R$  of 1.95 min) and is accompanied by peak tailing, but with improved sensitivity. The Thermo Scientific<sup>TM</sup> Hypercarb<sup>TM</sup> Porous Graphitic Carbon LC column, on the other hand, demonstrated a peak elution at  $t_R$  of 2.91 min, and the least sensitivity obtained under the tested analysis condition. Despite Hypercarb's reputation for exceptional retention of highly polar analytes, an early elution of  $\text{BrO}_3^-$  at a  $t_R$  of 1.61 min within a 10 min analysis run was previously reported (Anastassiades et al., 2021) using the Hypercarb ( $100 \text{ mm} \times 2.1 \text{ mm}$ ,  $5 \mu\text{m}$ ) column and Hypercarb Guard column ( $100 \text{ mm} \times 2.1 \text{ mm}$ ,  $5 \mu\text{m}$ ) (Table S2). The Phenomenex Synergi<sup>TM</sup> Polar-RP (biphenyl column)  $80 \text{ \AA}$  LC column exhibited satisfactory  $t_R$  at 6.64 min and a notable sensitivity improvement, establishing itself as the best-performing column under the tested condition. Interestingly, the Phenomenex Luna Silica (2) column  $100 \text{ \AA}$  ( $150 \text{ mm} \times 2.0 \text{ mm}$ ,  $5 \mu\text{m}$ ), previously reported by (Xian et al., 2017), demonstrated efficient retention for  $\text{BrO}_3^-$  at a  $t_R$  of 3.14 min within a total analysis run time of 6 min (Table S2).

Further optimization of the sensitivity and peak characteristics achieved by the Phenomenex Synergi<sup>TM</sup> Polar-RP column was investigated by testing various injection volumes of 1, 5, and  $10 \mu\text{L}$  using a spiked sample at a concentration level of  $2 \mu\text{g kg}^{-1}$ . The obtained results demonstrated that both peak intensity and resolution increased with increasing injection volume up to  $5 \mu\text{L}$ . However, beyond  $5 \mu\text{L}$ , column overload was observed, resulting in irreproducible peak intensities and a significant loss of resolution. Consequently, an injection volume of  $5 \mu\text{L}$  was chosen for optimal separation performance.

Regarding the analysis run time, the present study as well as the previously reported protocols (Anastassiades et al., 2021; Dong et al., 2019; Xian et al., 2017; Yokota et al., 2012) have all achieved complete elution within 6 to 15 min (Table S2). Notably, our proposed assay protocol and the separation method reported by (Xian et al., 2017) minimize the consumption of mobile phase solvents during the analysis run for each sample. Fig. 2 demonstrates the chromatographic separation

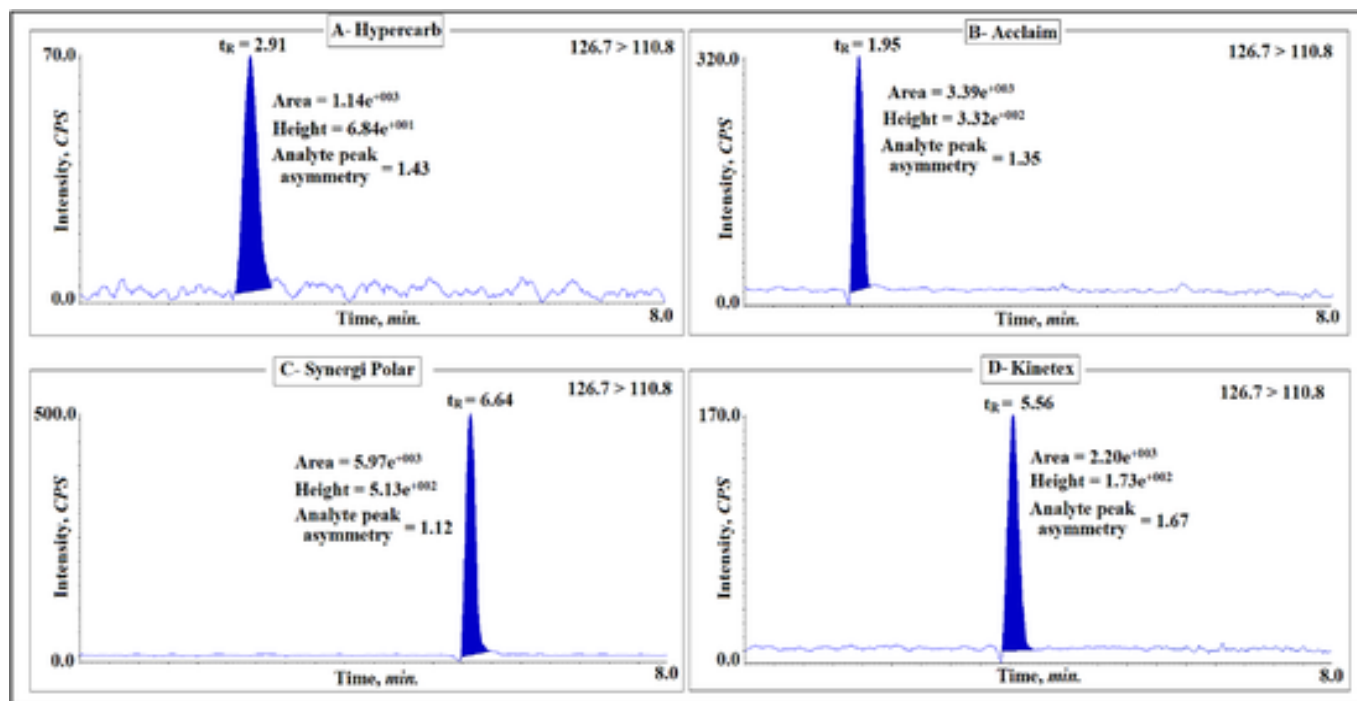


Fig. 2. Comparison of the  $\text{BrO}_3^-$  performance on four different candidate columns a) Thermo Scientific™ Hypercarb™ Porous Graphitic Carbon LC Column (100 mm × 2.1 mm, 5 μm), b) Acclaim™ RSLC Polar Advantage II 120 Å column (75 mm × 3.0 mm, 3 μm), c) Phenomenex Synergi™ Polar-RP (biphenyl column) 80 Å LC column (250 × 4.6 mm, 4 μm), d) Kinetex XB-C18 (core shell column) 100 Å column (150 mm × 4.6 mm, 3.5 μm) using 2 ng g<sup>-1</sup> spiking samples.

efficiency of the studied  $\text{BrO}_3^-$  on four different columns provided by various suppliers.

### 3.4. Sample preparation optimization

#### 3.4.1. Sample size and dilution magnitude

Appropriate selection of the proper sample size and the dilution factor is essential for developing an efficient sample preparation protocol capable of providing reliable test results from representative analytical samples. Therefore, sample sizes of 2.5, and 5 g were spiked at a concentration level of 2 μg kg<sup>-1</sup> in triplicates and subsequently extracted with a various volumes of MeCN-water (4:1, v/v), representing a dilution factor of 2× to 10×, each. Interestingly, at 4× and 5×, higher recovery percentages were achieved ranging from 87% to 90.5%, with CV% < 11%, in addition to minimal introduction of interfering components (tolerable ME) for each tested sample size. On the other hand, low recovery percentages of < 55% and intermediate signal suppressions of -36% and less than -50% were obtained at 2×, each. For each tested portion at 3× dilution level, however, a fair good recovery nearby 74% were obtained, an intermediate signal suppressions were observed. Despite the fact that, at 10× dilution level, high recoveries and tolerable ME were obtained, it was not selected owing to the inability to achieve lower LOQ levels as will be described later.

It has been decided not to perform testing for small samples sizes such as 0.5 g and 1 g test portions so as to avoid any possible fluctuations that may arise at very low levels of determinations. From this point of view, and in order to select a representative analytical sample, a sample size of 5 g was found appropriate for conducting  $\text{BrO}_3^-$  testing. In addition, a dilution magnitude of 4× was deemed suitable for the efficient  $\text{BrO}_3^-$  extraction from the preliminary and bakery products while maintaining tolerable MEs, and attaining lower LOD and LOQ levels. This could be due to the optimal achieved ratio between the proposed sample size and the optimized volume of the employed extraction

solvent, leading to maximum available surface area for efficient extraction.

#### 3.4.2. Extraction optimization and matrix effect study

Initially, a commonly used extraction solvents such as MeOH and MeCN were tested at different mixing ratios with water (1:1, 3:2, 7:3, 4:1, and 9:1, v/v) for direct extraction of the anionic polar  $\text{BrO}_3^-$  from the preliminary and bakery products. All samples were spiked at 2 μg kg<sup>-1</sup> and analyzed in triplicates using mechanical shaker, and heat-assisted extraction techniques like ultrasonic-assisted extraction and heated water bath with a horizontal mechanical shaker at various time intervals and/or temperatures as will be discussed later.

Recovery percentages as well as the MEs obtained owing to the direct extraction with MeOH and all of its corresponding mixtures with water exhibited inconvenient results. Whereas, the methanolic extracts yielded poor recoveries of only 20% and was associated with an intermediate ME of signal suppression at -46.6%. Besides, all remaining mixtures also provided poor recovery results in the range of 32.1% to 43%. Matrix effect results obtained showed that whatever the mixing ratio with water is, ME has not been mitigated, but a strong signal suppression was observed in the range of -70.4% to -55%. This may explain why (Anastassiades et al., 2021) incorporated a quantitation approach based on an isotopically labeled internal standard to correct for any losses in recovery percentages and strong signal suppression effects on the accuracy of the results obtained after conducting extraction with a mixture of MeOH-water (1:1, v/v) (Table S2). Additionally, a comparison of all presented LOQ values, including ours, exhibited that (Anastassiades et al., 2021) reported the highest LOQ value, as this will be illustrated later (Table S2).

Similarly, and as shown in Fig. 3, MeCN extracts demonstrated poor recoveries at levels below 55% under all employed extraction-assisting techniques. Increasing up the water mixing ratio with MeCN till (4:1, v/v) has resulted in ameliorating the recoveries obtained. At MeCN-water (4:1, v/v), optimal results were successfully achieved reaching a recov-

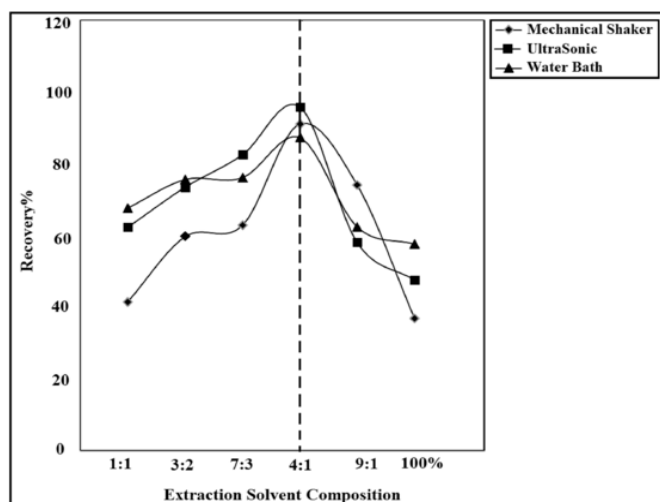


Fig. 3. Relationship between different extraction solvent composition ratios of MeCN-water and the obtained recovery percentages through different extraction-assisting techniques a) mechanical shaker; b) ultrasonic; and c) water bath.

ery percentage of approximately 90% with all employed extraction-assisting techniques.

Any further increase in the water ratio has exhibited a significant reduction in the recovery percentages obtained to levels below 80% till approximately 40%. In terms of MEs obtained, and as shown in Fig. 4, MeCN extracts showed intermediate signal enhancement while a signal suppression of intermediate to strong effects were recorded for MeCN-water mixtures of (7:3, 3:2, and 1:1, v/v) at the range of -80% to -40%. On the contrary, tolerable MEs were achieved at the respective mixing ratios of (9:1 and 4:1, v/v). It should be noted that under all tested extraction-assisting techniques, both investigated recovery percentages and MEs showed comparable results at each tested extraction condition. Therefore, it could be concluded that the employed extraction-assisting techniques have no a significant effect on the outputs obtained, but only the solvent composition tested. Nevertheless, an extraction mixture of MeCN-water (4:1, v/v) was selected to undergo an efficient  $\text{BrO}_3^-$  direct extraction from samples of preliminary and bakery products. This is because of the acceptable recoveries achieved in conjunction with perfect tolerable ME obtained.

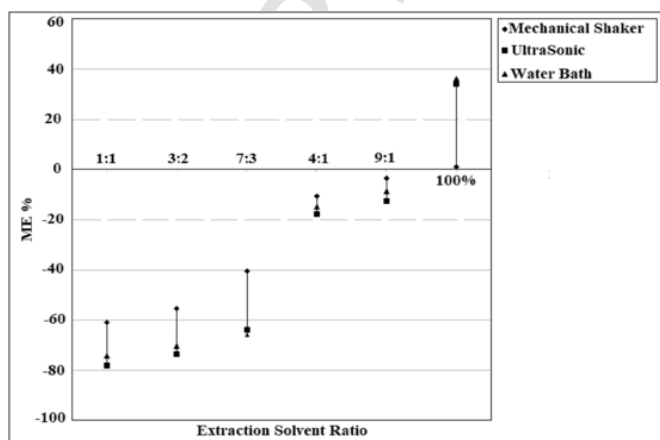


Fig. 4. Relationship between distinct MeCN-water extraction solvent composition ratios and ME% achieved via various techniques: a) mechanical shaker; b) ultrasonic; and c) water bath.

In continuous context, a mixture of MeCN-water (1:1, v/v) has been reported by (Xian et al., 2017) for direct extraction of  $\text{BrO}_3^-$  from flour samples using ultrasonic-assisted extraction in 10 min. However, an essential clean-up procedures were found mandatory to control the ME. Thus, a post clean-up procedure with 100 mg of C18 sorbent and 50 mg of GCB sorbent was performed resulting in tolerable MEs at levels of -11%. Despite the fact that the our proposed extraction protocol yielded comparable recoveries to that obtained by (Xian et al., 2017) (85.7 to 90.5%), a streamlined protocol applying a solid-liquid extraction procedures was successfully achieved negating any additional procedures (Table S2).

Likewise, a recent study was also reported by (Dong et al., 2019) for extracting  $\text{BrO}_3^-$  using ultrasonic-assisted extraction in 20 min by an acidified MeCN with 1% formic acid, but from fruits and vegetables samples. This called for involvement of additional procedures such as solvent exchange step following a gentle nitrogen stream evaporation just prior to a clean-up step based on a modified QuEChERS protocol employing 100 mg of C18 and 40 mg of GCB. The overall recoveries reported were in the range of 86.6 to 99.5%, with a tolerable ME% of -10.2% (Table S2).

#### 3.4.3. Impact of extraction-assisting techniques on $\text{BrO}_3^-$ extraction efficiency

The role of applied extraction-assisting and phase-out separation techniques in attaining high recoveries and tolerable MEs was studied using water bath with mechanical shaker, ultrasonic, and mechanical shaker along with applied centrifugation force/time. All influential parameters such as temperature and the contact time between the applied extraction solvent and the target matrix on both recovery percentages and MEs were assessed.

For instance, in heat-assisted extraction applications, samples extracted with a mixture of MeCN-water (4:1, v/v) using mild shaking conditions (110 rpm) at different time intervals of 1, 5, 10, 15, 20, 25, and 30 min by a heated water bath at 40, 50, and 60 °C. Regardless of the tested temperatures, the obtained results demonstrated that optimal extraction efficiencies have been achieved at an extraction time of 20 min. At the same time intervals, however, a temperature is induced during the ultrasonic-assisted extraction, comparable results were obtained in a duration of 20 min. The obtained results successfully demonstrated an achievement for acceptable recovery percentages of 82% and 90%, and tolerable MEs of -14.7% and -17.8% by both techniques tested, respectively.

On the other hand, and at a similar time intervals, extraction-assisted with vertical mechanical shaking at mild, moderate and strong strokes of 500, 700, and 1000 rpm was respectively tested at the ambient temperature. At 20 min, optimal extraction efficiencies were successfully achieved with moderate strokes of 700 rpm where acceptable recovery percentages of 87.5% along with tolerable ME results (-10.6%) were achieved (Fig. S2). Similarly, both mild and strong vertical mechanical shaking has resulted in acceptable recovery percentages at 71% and 89%, respectively, but with MEs of intermediate signal suppressions. At time intervals below 20 min, a significant decrease in the resultant recoveries associated with MEs of intermediate signal suppressions is observed. It should be noted that all tested extraction techniques have resulted in additional matrix component transfer to the extraction solvent, expressed as ME of intermediate signal suppression, at time intervals exceeding 20 min.

It is noteworthy that the application of various extraction-assisting techniques enabled efficient  $\text{BrO}_3^-$  residue extraction in only 20 min, although different strokes and temperature combinations were employed. Regardless of the temperature tested or induced by the ultrasonic system's mechanical waves, the efficiency of  $\text{BrO}_3^-$  residue extraction mirrored that of the mechanical shaker's moderate strokes at ambient temperature.

### 3.4.4. Sample purification

Following the efficient partitioning of the studied analyte to the extraction solvent, complete phase-out separation is a critical step in obtaining clear extracts with the least amount of interferences. In this regard, and in order to reduce any possible competition originates from interfering substances to the binding sites on the extraction phase, a combination of centrifugation force and time has been studied under cooling conditions as well as after samples being kept overnight under deep freezing conditions at a temperature of  $-20 \pm 2$  °C so as to facilitate removal of solidified lipids and precipitated proteins. Consequently, various centrifugation forces of 6000, 9000, 12,000, and 16,020 rcf were applied at different time intervals of 1, 5, 10, 15, 20, 25, and 30 min, each.

At a centrifugation force of 16,020 rcf, it was found that by increasing the centrifugation time, a significant reduction in the MEs obtained was observed until reaching 15 min. Any further centrifugation times applied had no tangible effect on the resultant MEs. All other tested centrifugation speeds below 16,020 rcf yielded extracts with additional matrix components over a tested duration period ranging from 1 to 15 min, where an intermediate signal suppression was obtained. Otherwise, both tested centrifugation speeds of 9000 and 12,000 rcf have provided tolerable MEs at times exceeding 20 min (Fig. 5a).

Surprisingly, at whatever centrifugation force/time applied, complete phase-out separation of clean extracts associated with tolerable MEs was successfully achieved following the overnight deep freezing-out condition for the tested samples. As a quite similar slopes for all studied conditions (various centrifugation force/time versus ME%) were obtained (Fig. 5b). This might be due to the additional precipitation of poorly soluble constituents such as protein, carbohydrates, and sparingly soluble minerals in the applied extraction solvent, leading to sufficient purification and cleanliness of the final extracts. This indicates the superior role of deep freezing-out conditions over the applied centrifugation forces/times in efficient removal of interfering substances that may hinder accurate  $\text{BrO}_3^-$  quantitation. Nevertheless, and based on the obtained results, complete phase-out separation associated with minimal MEs possible was preferred to be carried out using a cen-

trifugation speed of 16,020 rcf for 15 min under cooling conditions rather than the time-consuming procedure of the overnight deep freezing-out applications. Fig. 5 demonstrates the relationship between centrifugation force/time or the overnight deep freezing-out conditions and the resultant MEs of  $\text{BrO}_3^-$  extracted from Baladi bread samples with a mixture of MeCN-water (4:1, v/v).

In all, the optimized extraction procedure is performing well for  $\text{BrO}_3^-$  analysis in preliminary and bakery products when a representative sample size of 5 g is efficiently extracted with a mixture of 20 mL MeCN-water (4:1, v/v) (corresponding to a dilution magnitude of 4×) utilizing a moderate vertical stroke of 700 rpm by the mechanical shaker. Clear extracts were subsequently obtained with high centrifugation speed of 16,020 rcf for 15 min under cooling conditions followed by direct injection into LC-ESI-MS/MS in negative ion-mode.

Compared to previous methods, our optimized assay protocol enables exceptional  $\text{BrO}_3^-$  determination in diverse commodities using a streamlined “solid-liquid extraction” procedure coupled with LC-ESI-MS/MS, achieving an overall analysis time of just 35 min. This represents a significant improvement over previously reported analysis times, which typically range from 50 to 105 min for  $\text{BrO}_3^-$  analysis in fresh foods, fruits, vegetables, and flour samples using various extraction and determination techniques such as SLE and SPE coupled with IC-MS (Aggrawal & Rohrer, 2020), modified QuEChERS protocol coupled with LC-ESI-MS/MS (Dong et al., 2019), and SLE combined with HPLC with a post-column derivatization (Yokota et al., 2012) (Table S2). Notably, our method achieves comparable overall analysis times to those reported for modified QuEChERS coupled with LC-ESI-MS/MS for flour testing (Xian et al., 2017) and SLE combined with LC-ESI-MS/MS for lettuce varieties (Anastassiades et al., 2021) (Table S2).

## 4. Statistical analysis-guided selection of extraction mixture composition

For in-depth evaluation and proper selection of the applied extraction mixture and extraction-assisting techniques for efficient  $\text{BrO}_3^-$  extraction from preliminary and bakery products, statistical analysis was

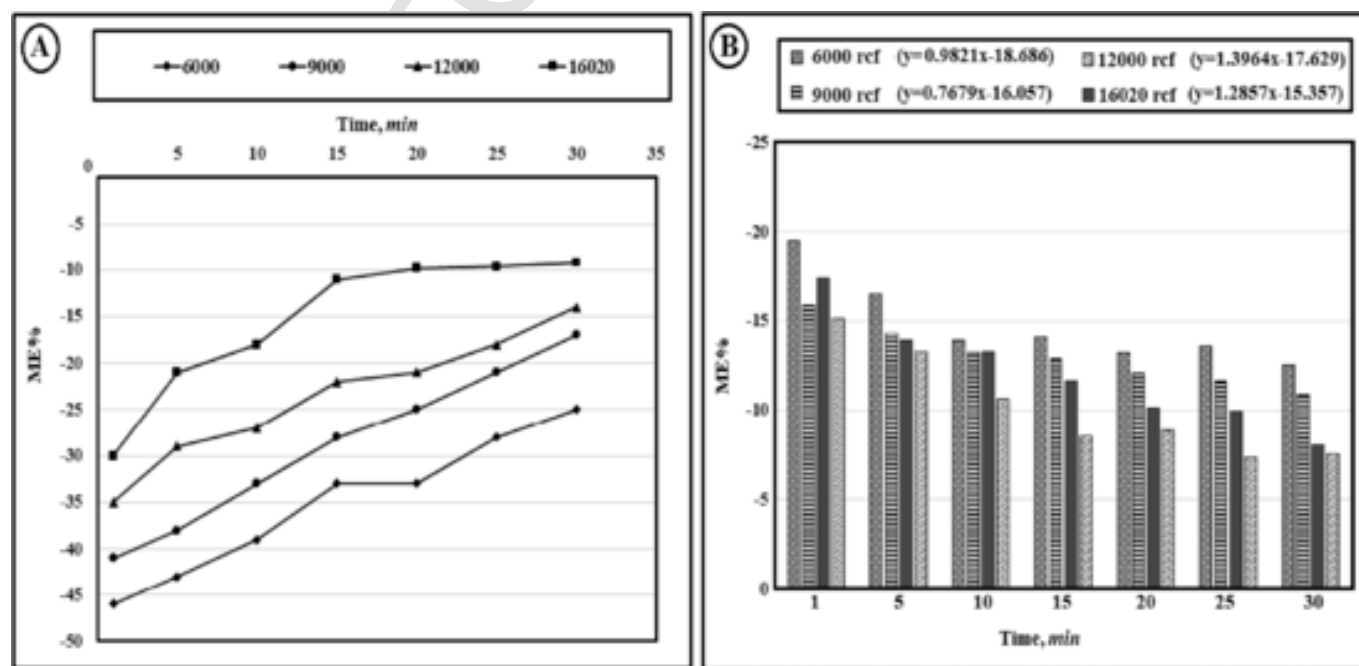


Fig. 5. The relationship between a) cooling centrifugation force/time; b) the overnight deep freezing-out conditions and the resultant matrix effects of  $\text{BrO}_3^-$  residues extracted from Baladi bread samples with a mixture of MeCN-water (4:1, v/v).



performed using one-way ANOVA and descriptive statistics. As shown in **Table S3a**, results of one-way ANOVA testing exhibited a significant difference among the average recovery percentages obtained ( $p < 0.05$ ). This indicates that variable and inconsistent recoveries have been emerged owing to the tested combinations of the applied extraction mixtures and techniques.

Thereby, further statistical evaluation was performed using the descriptive analysis to figure out which extraction mixture would provide better and meaningful results. In this sense, and for all employed extraction techniques, a mixture of MeCN-water (4:1, v/v) has provided an efficient  $\text{BrO}_3^-$  extraction. In which, the attained mean recovery percentages, median, standard deviation, S.E., and confidence level at 95% were 85.8%, 85.5%, 4.01, 2.31, and 9.96, respectively (**Table S3b**). On the other hand, all other tested extraction mixtures provided a relatively low mean recovery percentages ranging from 44.3% to 69.3%, and was associated with high data scatter in terms of the resultant errors. In which the obtained S-Es were in the range of 4.48 to 7.49, while the confidence levels obtained at 95% were ranging from 19.29 to 32.22.

Moreover, further one-way ANOVA and descriptive statistical analyses were carried out to describe whether differences in the recoveries obtained (5 replicates, each) with an extraction mixture of MeCN-water (4:1, v/v) by the applied extraction techniques are significant or not. It was found that all applied extraction techniques have provided comparable recoveries where the ANOVA test revealed non-significant results ( $p > 0.05$ ) (**Table S4a**). In addition, a matched recovery percentages were also confirmed via further evaluation by descriptive statistics for all obtained results extraction-assisting techniques tested. In terms of acceptable mean recovery percentages obtained along with the minimal associated errors and intervals, it could be undoubtedly concluded that all applied extraction techniques under the described analysis conditions would provide exceptional performance (**Table S4b**). However, we decided to use the mechanical shaking-assisted extraction for efficient and practically convenient extraction procedures of  $\text{BrO}_3^-$  residues from preliminary and bakery products with a mixture of MeCN-water (4:1, v/v) as it provided perfect tolerable MEs as described earlier.

## 5. Method validation

### 5.1. Selectivity

The proposed method of analysis was conducted on flour and bread samples as representative of preliminary and bakery products, respectively. To assess the selectivity of the test method, spike samples were prepared at a concentration level of  $0.2 \mu\text{g kg}^{-1}$ , equivalent to the LOQ value, and compared to blank samples. The obtained results indicated

that the studied compound can be accurately detected and quantified without any interference from other substances in the targeted elution region. **Fig. S3** presents a comparison of the spiked sample with  $\text{BrO}_3^-$  at the LOQ level to the blank sample using the proposed assay protocol.

### 5.2. Linearity range

A Multilevel calibration curve composed of a set of 6 points ranging from 0.05 to  $100 \text{ ng mL}^{-1}$  was successfully achieved. Under linear regression equation of  $y = 1.07e^{+004}x + 4.19e^{+003}$ , a linear regression coefficient ( $R^2$ ) of 0.9999 was obtained (**Table 1**). Consequently, an accurate quantitation results in real applications as well as in a quality control samples analysis and external quality control testing via PT analysis is confirmed. Owing to the wide-range linearity achieved, it would be possible undergo a direct quantitation for tested positive samples with high concentration levels without any need for further dilutions. The method linearity was also investigated using spiked samples at the studied validation levels (0.2, 1, 2, and  $10 \mu\text{g kg}^{-1}$ , 10 replicates each). Results confirmed the test method linearity, with an  $R^2$  of 0.9999 and 0.9995 for flour and Baladi bread spiked samples.

As shown in **Table S2**, and in agreement with the previously reported protocols (Aggrawal & Rohrer, 2020; Dong et al., 2019; H. Shi & Adams, 2009; Xian et al., 2017), a wide linear dynamic range was successfully achieved. Otherwise, only the current study as well as the developed test method by (Anastassiades et al., 2021) for  $\text{BrO}_3^-$  analysis in lettuce varieties have successfully reported levels as much as low at a concentration of  $0.05 \text{ ng mL}^{-1}$ . On the contrary, compared to our proposed assay protocol, a narrow linear dynamic range at a concentration range of  $0.05\text{--}10 \text{ ng mL}^{-1}$  and  $1\text{--}20 \text{ ng mL}^{-1}$  have been respectively demonstrated for  $\text{BrO}_3^-$  analysis in fresh foods by (Anastassiades et al., 2021) and (Yokota et al., 2012). Although, both (Xian et al., 2017) and (Aggrawal & Rohrer, 2020) demonstrated a wide range of linearity at  $1.5\text{--}250 \text{ ng mL}^{-1}$  and  $0.5\text{--}500 \text{ ng mL}^{-1}$  for  $\text{BrO}_3^-$  analysis in flour samples, our proposed assay protocol presented levels as far less 10 to 30 times the lowest reported level. **Table 1** exhibit all necessary validation parameters obtained values as per Eurachem 2014 validation guideline.

### 5.3. LOD and LOQ

This study achieved a practical LOD of  $0.015 \mu\text{g kg}^{-1}$  and a practical LOQ of  $0.05 \mu\text{g kg}^{-1}$  for  $\text{BrO}_3^-$  in both tested commodities (**Table 1**). These values are significantly lower than those reported in previous methods (Aggrawal & Rohrer, 2020; Anastassiades et al., 2021; Dong et al., 2019; H. Shi & Adams, 2009; Xian et al., 2017). Whereas our assay is 33 to 333 times more sensitive for LOD and 120 to 400 times more sensitive for LOQ (**Table S2**). Notably, our method also shows exceptional sensitivity, achieving a 7-fold lower LOD (Zuo et al., 2023) and a

**Table 1**

Results of validation requirements as per the Eurachem 2014 guideline using the proposed assay protocol for bromate additive use on two representative commodities, including a) preliminary products (e.g., flour) and b) bakery products (e.g., Baladi bread)

Spiking level, $\mu\text{g kg}^{-1}$	Mean recovery % $\pm$ SD	Repeatability (n = 10 replicates, each)		Intermediate precision at $0.2 \mu\text{g kg}^{-1}$ (n = 30 replicates)		Linear dynamic range, $\text{ng mL}^{-1}$	$R^2$	LOD, $\text{ng g}^{-1}$	LOQ, $\text{ng g}^{-1}$	MU%
		Mean Conc., $\mu\text{g kg}^{-1}$	RSD	Mean Conc., $\mu\text{g kg}^{-1}$	RSD					
Preliminary products (e.g., Flour)										
0.2	$91.0 \pm 7.6$	0.18	8.20	0.198	9.64	0.05–100	0.9999	0.015	0.050	< 28
1	$102.0 \pm 4.0$	0.99	3.88							
2	$92.0 \pm 3.5$	1.94	3.68							
10	$92.0 \pm 2.6$	9.30	2.93							
Bakery products (e.g., Baladi bread)										
0.2	$96.3 \pm 8.2$	0.19	8.60	0.187	10.10	0.05–100	0.9999	0.015	0.050	< 29
1	$93.6 \pm 9.4$	0.94	9.80							
2	$103.0 \pm 8.4$	2.08	8.05							
10	$88.6 \pm 6.1$	8.92	6.90							

6–12-fold lower LOQ (Yokota et al., 2012; Zuo et al., 2023) compared to previously reported estimated values for various commodities, including water and fresh foods (Table S2). This will ensure effective regular oversight for this banned analyte in preliminary and bakery products in accordance with the EU regulations (European Parliament and the Council of the European Union, 2008) and the recently promulgated limits in binding technical rules issued by the national regulatory agency (NFSA, National Food Safety Authority, 2020).

#### 5.4. Trueness and precision

The trueness of the test method was expressed in terms of recovery percentages  $\pm$  SD. Where representative commodities to preliminary and bakery products such as flour and Baladi bread were respectively tested. Blank samples of both tested commodities were spiked with  $\text{BrO}_3^-$  standard at concentration levels of 0.2, 1, 2, and 10  $\mu\text{g kg}^{-1}$  and subsequently analyzed using the proposed assay protocol ( $n = 10$  replicates, each). For flour samples, the average recoveries obtained ranged from 91 to 102%, with SDs ranging from  $\pm 2.6$  to  $\pm 7.6$ . Baladi bread, on the other hand, has demonstrated an average recovery of 88.6% to 103%, with SDs ranging from  $\pm 6.1$  to  $\pm 9.4$  (Table 1).

Further confirmation of the trueness results was carried out by analyzing two PT samples of water samples supplied by FAPAS (round no. DWC086, June 2022) and (round no. DWC089, August 2022). Each sample was analyzed in triplicates and expressed as average concentration  $\pm$  SD. The average concentrations obtained were  $15.5 \pm 0.003$  and  $9.5 \pm 0.002 \mu\text{g kg}^{-1}$  for both tested PT samples, respectively. According to the FAPAS report, the reported results were compared to the assigned value and deemed satisfactory as the z-score results were in between  $\pm 2$  (Table S5).

In terms of precision, for flour samples, CV% obtained results for both repeatability and intermediate precision were 2.93% to 8.20%, and 9.64%, respectively. For Baladi bread samples, CV% results of repeatability and intermediate precision were 6.9% to 9.8%, and 10.1%, respectively (Table 1). Overall, and as shown in Table S2, both results achieved in our assay protocol and the previously reported protocols were well below the EU regulatory requirements (European Commission, 2021) concerning the CV% results of repeated analysis for spiked or incurred samples. In accordance with Eurachem guidelines, CITAC Guide CG 4 (Ellison & Williams, 2012), the expanded uncertainty at a 95% confidence level and a coverage factor (K) of  $2 \times$  combined uncertainty was respectively  $< 28\%$  and  $29\%$  for both flour and Baladi bread samples (Table 1).

## 6. Application

In order to investigate the applicability of the validated assay protocol and to confirm its practicality and effectiveness in real sample analysis,  $\text{BrO}_3^-$  residues were tested in 288 samples collected from the domestic market in Egypt. Out of the analyzed preliminary products, only one sample of baking powder products (3.8%) was tested positive at a concentration level of  $6.3 \mu\text{g kg}^{-1}$ . All other preliminary products including flour and cake powder samples were found devoid from any  $\text{BrO}_3^-$  residues.

On the other hand, except for Baladi bread, soft baguettes, bread rolls, pizza, pate, and pizza crust, all remaining bakery products showed negative results for  $\text{BrO}_3^-$  residues. Results obtained have revealed that the overall concentration range for bakery products tested positive samples were 1.7–72.2  $\mu\text{g kg}^{-1}$ . Respective to the EU and the national regulation (European Parliament and the Council of the European Union, 2008; NFSA, National Food Safety Authority, 2020), a 50% of the analyzed Baladi bread products (19 samples) were violating the established limits at a concentration range of 2.3 to 72.2  $\mu\text{g kg}^{-1}$ . Another high violation rates were also observed in pate products, where 42.9% of the samples (12) were detected positive presenting residue

concentration range of 1.7 to 29.8  $\mu\text{g kg}^{-1}$ . In addition, bread rolls have also demonstrated a high violation rate at 22.2%, where 4 tested positive samples were found to have a  $\text{BrO}_3^-$  residue in a concentration range of 2.2–7.6  $\mu\text{g kg}^{-1}$ . A few samples of soft baguettes (2 products) and pizza products (1 sample) exhibited positive results for  $\text{BrO}_3^-$  residue, where percentages of violation observed were only 5.3% and 9.1%, respectively. Soft baguettes detected concentration range was 2.8–11.5  $\mu\text{g kg}^{-1}$ , while pizza's obtained concentration was 7.2  $\mu\text{g kg}^{-1}$ .

While negative results were obtained for flour samples in the present study, it is noteworthy that several samples among the 50 tested flour products yielded positive results at levels below the LOQ value of 2  $\mu\text{g kg}^{-1}$ , as reported by (Xian et al., 2017). Another study conducted by (Y. Shi et al., 2006) have tested ordinary and treated flour samples, noodles, bread, and steamed bread. The reported results revealed that  $\text{BrO}_3^-$  residue was not identified in the ordinary flour and steamed bread, but different concentrations were detected in the remaining commodities. Treated flour samples exhibited  $\text{BrO}_3^-$  concentration in the range of 140.1 to 202.3  $\mu\text{g kg}^{-1}$ , as well as the samples of bread and noodles products presented concentrations of 84.0 and 34.4  $\mu\text{g kg}^{-1}$ , respectively. In this regard, it was found that our findings provide comparable results to that had been obtained by (Y. Shi et al., 2006) for  $\text{BrO}_3^-$  concentration levels in bread samples.

In conclusion, the obtained results confirm the applicability of the validated assay protocol to effectively analyze  $\text{BrO}_3^-$  residue in preliminary and bakery products. Moreover, safety levels assessment along with regular oversight of the domestic market by the national regulatory agencies is highly recommended. Table 2 presents the prevalence of  $\text{BrO}_3^-$  residue in preliminary and bakery products collected from the domestic market in Egypt.

To further confirm our proposed method's effectiveness and to ensure other extraction techniques are working properly, all samples identified as positive were re-analyzed using optimized extraction protocols of ultrasonication and a water bath with mechanical shaking. As shown in Fig. S4, all processing methods yielded similar results, with an SD  $< 14$ . This demonstrates that all optimized sample processing procedures provide reliable  $\text{BrO}_3^-$  residue analysis results.

## 7. Conclusion

A straightforward solid-liquid extraction protocol was developed using an optimized MeCN-water mixture (4:1, v/v). This method, combined with LC-ESI-MS/MS, enabled efficient and accurate determination of  $\text{BrO}_3^-$  in preliminary and bakery products at ultra-low concentrations. Bromate-efficient extraction relies on two key factors: choosing the right extraction solvent composition and the success of the physical cleanup procedure, regardless of the extraction-assisting techniques used. This assay protocol is significantly more sensitive than existing methods, achieving a 7-fold lower LOD and a 6–12-fold lower LOQ for  $\text{BrO}_3^-$  residue analysis in various food products. The validated method applies to real and PT samples, improving sample processing capacities in routine work laboratories. It can also be used by regulatory agencies for regular  $\text{BrO}_3^-$  oversight, ensuring consumer safety in Egypt.

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## CRediT authorship contribution statement

**Amira A. Abdulrahman El-Bahar:** Writing – original draft, Validation, Methodology. **Amr H. Shendy:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Alaa El-Dean Fathy Ahmed Aboalhasen:** Methodology. **Ahmed M. Gomma:** Writing – review & editing, Supervision, Conceptualization.

Table 2

Prevalence of BrO<sub>3</sub><sup>-</sup> residues in preliminary and bakery products collected from the domestic market in Egypt.

Category	Commodity tested	Bromate residue level				
		No. of tested samples	No. of detected positive samples	Concentration range, µg kg <sup>-1</sup>	% of Violating samples <sup>a</sup>	Overall violation <sup>b</sup>
Preliminary products	Flour	22	N·D	N·D	0%	0%
	Baking powder	3	1	6.3	33.3%	3.8%
	Cake powder	1	N·D	N·D	0%	0%
Total tested preliminary samples and overall violation%		26	1	6.3	3.8%	
Bakery products	Bread	38	19	2.3–72.2	50%	7.25%
	Hot dog buns (Soft baguettes)	38	2	2.8–11.5	5.3%	0.76%
	Cake	39	N·D	N·D	0%	0%
	Croissant	11	N·D	N·D	0%	0%
	Bread rolls	18	4	2.2–7.6	22.2%	1.53%
	Pizza	11	1	7.2	9.1%	0.38%
	Pate	28	12	1.7–29.8	42.9%	4.58%
	Toast	9	N·D	N·D	0%	0%
	Noodles	14	N·D	N·D	0%	0%
	Biscuits	28	N·D	N·D	0%	0%
	Flour wraps	17	N·D	N·D	0%	0%
	Pizza crust	1	1	9.6	100%	0.38%
	Burger bun	8	N·D	N·D	0%	0%
	Bread stick	2	N·D	N·D	0%	0%
Total tested samples of bakery products and overall violation%		262	39	1.7–72.2	14.9%	0.38%–7.25%
Gross total		288	40	1.7–72.2	13.9%	

Eman H. Ismail: Writing – review & editing, Supervision, Conceptualization.

#### Declaration of competing interest

The authors declare that they are not aware of any financial or interpersonal issues that might have appeared to have an impact on the study that was the subject of this work.

#### Data availability

Data will be made available on request.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2024.139416>.

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