The Cambodia Journal of Basic and Applied Research 7(Spl) 2025

Print ISSN: 2790-3508 Online ISSN: 2790-3516

The Cambodia Journal of Basic and Applied Research

journal homepage: https://cjbar.rupp.edu.kh

Determination of Benzoic Acid and Sorbic Acid in Fish Sauce Using HPLC-UV

TOM Sakmay[1,2], PHAL Sereilakhena[2,3*]

[1]Science Technology and Innovation National Laboratory (STINL), Ministry of Industry, Science, Technology and Innovation (MISTI), Cambodia.
[2]Graduate Program in Chemistry, Graduate School of Science (GSS), Royal University of Phnom Penh (RUPP), Russian Federation Boulevard, Khan Toul Kork, Phnom Penh, Cambodia.
[3]Department of Chemistry, Royal University of Phnom Penh (RUPP), Russian Federation Boulevard, Khan Toul Kork, Phnom Penh, Cambodia.

ARTICLE INFO
Editorial responsibility: Prof. CHEY Chan Oeurn
Received: November 04, 2024
1st Revised: 11 January, 2025
2nd Revised: 31 January 2025
Accepted: 04 February 2025
Published online: 09 April 2025

Keywords:
Benzoic acid,
Sorbic acid,
Preservative,
HPLC-UV,
Potassium ferrocyanide,
Zinc acetate,
Fish sauce

សង្ខិត្តន័យ

សារធាតុថែរក្សាចំនួនពីរគឺអាស៊ីតបង់សូអ៊ិច (BA) និងអាស៊ីតសូរប៊ិក (SA) ត្រូវបានកំណត់បរិមាណនៅក្នុងទឹកត្រីដោយប្រើវិធីសាស្ត្រ HPLC-UV ។ ភាគសំណាកទឹកត្រីត្រូវបានរៀបចំមុនដោយប្រើ 2 mL នៃ 0.5 M នៃសង្កសីអាសេតាត $Zn(CH_3COO)_2$ និង 2 mL នៃ 0.25 M នៃប៉ូតាស្យូមហ្វេរ៉ូស្យានីត, $K_4[Fe(CN)_6]$ មុនពេលធ្វើការវិភាគ។ ការវិភាគត្រូវបានធ្វើដោយប្រើ HPLC-UV ដែលមានផាសចល័តជាល្បាយនៃមេតាណុល និងអាម៉ូញ៉ូមអាសេតាត (0.02 M, pH 6.75) ក្នុងសមាមាត្រជាមាឌ 20:80 ដោយដំណើរការជាអ៊ីសូក្រាទិច ជាមួយនឹងអត្រាលំហូរ 0.8 mL/min សីតុណ្ហភាពរបស់កូឡោនគឺ 30 °C និងដំហានរលក 220 nm ។ វិធីសាស្ត្រនេះបានផ្តល់លទ្ធផលលីនេអ៊ែរក្នុងចន្លោះ 25 - 200 mg/L សម្រាប់ BA និង SA ដែលមាន R^2 ស្មើ 0.9962 និង 0.9985 រៀងគ្នា។ LOD ដែលបានវាស់វែងគឺ 10 mg/L (S/N ≥3) ហើយ LOQ គឺ 30 mg/L (S/N ≥10) សម្រាប់ទាំង BA និង SA។ ទឹកត្រីចំនួន 21 មុខ រួមមាន ថៃ 6 ម៉ាក (T1-T6) វៀតណាម 4 ម៉ាក (V1-V4) និងកម្ពុជា 11 ម៉ាក (C1-C11) ត្រូវបានប្រមូលពីផ្សារដេប្រើនក្នុងរាជធានីភ្នំពេញ ដើម្បីពិនិត្យ។ វាត្រូវបានគេរកឃើញថា ទឹកត្រីចំនួន 7 (33.3% នៃសំណាកទាំងអស់) ដែលមាន 4 ម៉ាក ពីប្រទេសកម្ពុជា និង 3 ម៉ាក ពីប្រទេសវៀតណាម មានកម្រិត BA ចន្លោះពី 33.3 ± 0.4 និង 558 ± 2 mg/L ។ ទឹកត្រី 14 មុខ (6 ពីប្រទេសកម្ពុជា 2 ពីវៀតណាម និង 6 ពីប្រទេសថៃ) ដែលត្រូវនឹង 66.7% នៃសំណាកទាំងអស់ មាន SA ចាប់ពី 19.8 ± 0.7 (<LOQ) ដល់ 251 ± 2 mg/L ។ មានតែ C4, V2, និង V4 ដែលមានទាំង BA និង SA ប៉ុន្តែ C5, C9, និង V1 មិនអាចកំណត់រក BA និង SA បានទេ។ កម្រិតនៃ BA និង SA ដែលមាននៅក្នុងទឹកត្រីគឺទាបជាង 1000 mg/L ដែលជាកម្រិតអតិបរមាអនុញ្ញាតដោយ Codex Alimentarius

*Corresponding author: Department of Chemistry, Royal University of Phnom Penh (RUPP), Russian Federation Boulevard, Khan Toul Kork, Phnom Penh, Cambodia.
E-mail addresses: phal.sereilakhena@rupp.edu.kh (PHAL Sereilakhena)
DOI: https://www.doi.org/10.61945/cjbar.2025.7.spl.01.

Abstract

Two preservatives, benzoic acid (BA) and sorbic acid (SA), were quantified in the fish sauce using an HPLC-UV detector. The fish sauce sample was pre-treated using 2 mL of 0.5 M of zinc acetate, $Zn(CH_3COO)_2$, and 2 mL of 0.25 M of potassium ferrocyanide, $K_4[Fe(CN)_6]$, before analysis. The analysis was carried out using HPLC-UV under a mobile phase of methanol: ammonium acetate (0.02 M, pH 6.75) in a ratio 20:80, v/v operating in isocratic mode with 0.8 mL/min flow rate, column temperature of 30 ^oC, and wavelength of 220 nm. The method yielded linearity in the 25 – 200 mg/L range for BA and SA with R^2 of 0.9962 and 0.9985, respectively. The measured LOD was 10 mg/L (S/N ≥3) and LOQ was 30 mg/L (S/N ≥10) for both BA and SA. 21 fish sauce samples, comprising 6 Thai (T1-T6), 4 Vietnamese (V1-V4), and 11 Cambodian (C1-C11) brands, were gathered from several markets in Phnom Penh for examination. It was discovered that 7 fish sauces (33.3% of the samples), consisting of 4 brands from Cambodia and 3 from Vietnam, had BA levels between 33.3 ± 0.4 and 558 ± 2 mg/L. The 14 fish sauces (6 from Cambodia, 2 from Vietnam, and 6 from Thailand), 66.7% of the samples, containing SA ranged in concentration from 19.8 ± 0.7 (<LOQ) to 251 ± 2 mg/L. Only the C4, V2, and V4 contained both BA and SA, however, C5, C9, and V1 could not be detected for BA and SA. The levels of BA and SA found in the fish sauces are below the 1000 mg/L, maximum allowed by Codex Alimentarius.

Introduction

Consumption of food is fundamental for all living things because it provides energy for survival and all subsequent activities. But when food is stored for an extended period, rotting becomes the biggest concern. As a result, people implemented numerous measures to preserve their food supplies, including pickling, freezing, vacuum packaging, and the use of chemical preservatives (Amit et al., 2017). Food preservation refers to the methods used to handle and treat food in order to stop or slow down decomposition, enabling longer storage times and hence preventing food-borne illnesses (Zeuthen & Bøgh-Sørensen, 2003). Food preservation is among the earliest methods employed by humans to inhibit alterations induced by bacteria, enzymes, and physical forces (Mustafa et al., 2021).

Benzoic acid (BA) and sorbic acid (SA) are common preservatives used in food, beverages, toothpaste, cosmetics, and pharmaceuticals to control mold growth, stop germs from growing, and ward off various other dangers (Saad et al., 2005). However, using antiseptic chemicals excessively might be harmful to people's health. Codex Alimentarius' general standard for food additives states that the maximum amounts of sorbic and benzoic acids in fish sauce are 1000 mg/L (Codex, 1995). For benzoic acid and sorbic acid, the FAO/WHO Expert Committee on Food Additives suggests daily intakes of 5 and 25 mg/kg, respectively (Xu et al., 2019). As a result, it's critical to understand how much food products include preservatives such as sorbic and benzoic acids.

Analytical methods that have been used for the determination of benzoic acid (BA) and sorbic acid (SA) include high-performance liquid chromatography (HPLC) (Sirhan, 2018), capillary electrophoresis (CE) (Xu et al., 2019), gas chromatography (GC) (Kim et al., 2018), ultraviolet (UV) (Golge et al., 2015), thin-layer chromatography (TLC) (Saad et al., 2005), liquid chromatography coupled with mass spectrometry (LC-MS/MS) (Özdemir et al., 2020), biosensors (Golge et al., 2015; Özdemir et al., 2020), and lanthanide-sensitized luminescence (Burana-osot et al., 2014). Among those techniques, HPLC is the most common analytical method used because it offers high selectivity, requires less sample preparation, and does not require derivatization (Ammen & Al-Salhi, 2017).

Fish sauce (Toeuk Trey), Fig. 1, is a mixture of fermented fish and salt at a ratio of 3:1 (fish: salt) that is kept for several months up to a year (Chuon et al., 2014). It is a condiment used by over 90% of the population in Cambodia (Theary et al., 2013). The daily utilization of 15 to 30 mL/person is approximated to provide 7.5% of protein which is enough for its vitamin B12 content to prevent megaloblastic anemia (Thongthai & Gildberg, 2005). There have been reports of consumption of organic acid preservatives, i.e., benzoic and sorbic acid to delay the products' shelf life (Tungkijanansin et al., 2020). Therefore, ensuring the safety level of preservatives used in food is very important for consumers.

This study aims to determine the concentration of benzoic and sorbic acids in fish sauces using HPLC-UV. The 21 fish sauces were collected from different Phnom Penh markets and analyzed. The results of benzoic and sorbic acids obtained from all the fish sauce samples were compared with the permitted amount by Codex Alimentarius.

Conceptualizing benzoic acid and sorbic acid in sauces using HPLC-UV

Wen et al., developed and validated an in-tube solid-phase microextraction (SPME) with the HPLC-UV method to simultaneously determine benzoic and sorbic acids for 26 food samples including 10 kinds of sauces in China. However, the types of sauce samples were not specified. The diethylamine-modified poly (glycidyl methacrylate-co-ethylene dimethacrylate) monolithic capillary was employed as an extractant for benzoic and sorbic acids. The level of benzoic acid was found in 5 sauces from

Source: Authors' Photo

Fig. 1: Different types of fish sauces sold in Cambodia

417 to 930 mg/kg while SA was present in 3 sauces from 254 to 745 mg/kg, those amounts were lower than the permitted limit of 1000 mg/kg. None of the samples contained both acids (Wen et al., 2007).

An environment-friendly method of HPLC-UV was developed by researchers in China for the detection of benzoic and sorbic acids in soy sauce (Ding et al., 2015). The method gave excellent accuracy and precision with recovery of 96 to 104% and a relative standard deviation of 3%, respectively. Four soy sauces were analyzed by the developed method and found to have benzoic acid in all samples from 238.7 to 661.3 mg/kg and two of them had sorbic acid from 246.4 to 311.7 mg/kg, however, the found levels didn't exceed the legal limit.

Another research group in Malaysia also developed a reversed-phase HPLC method for the determination of benzoic and sorbic acid as well as methyl- and propylparabens in 67 foodstuffs including 15 sauce samples collected from supermarket in Kedah and Perlis of Peninsula Malaysia (Saad et al., 2005). The types of sauce samples were not specified. One sauce sample contained benzoic acid 1260 mg/kg which violated the limit level.

Benzoic and sorbic acids were evaluated in 54 samples bought from the supermarket in Tabriz City, Iran using dispersive liquid-liquid microextraction with HPLC-UV (Javanmardi et al., 2015). There were 15 ketchup sauces among the analyzed samples. Benzoic acid was found in ketchup sauces in the range of < 0.1 to 38.7 mg/kg while sorbic acid had in only one sauce sample with a concentration of 54.1 mg/kg. The Iranian and European legislation (European Commission 2011) prohibits consuming benzoic and sorbic acid as preservatives in ketchup sauces. However, the results reported by the researcher were just in small amount.

Air-assisted dispersive liquid-liquid extraction with organic phase solidification (AA-DLLME-OPS) was developed by a Russian researcher for the analysis of benzoic and sorbic acids in beverage and soy sauce samples using HPLC-UV (Timofeeva et al., 2019). Molten menthol was used as a dispersant to extract the analytes and provided high extraction efficiency. The method provided acceptable precision and accuracy. The benzoic and sorbic acids in soy sauces were lower than LOD of the method (0.03 mg/L for benzoic acid and 0.02 mg/L for sorbic acid).

According to the previous literature, there were no studies of benzoic and sorbic acid contents in fish sauce in Cambodia yet. Although some studies in other countries analyzed both acids in the sauces, the types of sauces were not specified, and still a lack of information on both acids in fish sauces. Therefore, determining both acids in fish sauces used in Cambodia is crucial for consumers.

Research Methodology

Chemicals and instrumentation. All of the chemicals used in this study were of analytical grade. Standard of benzoic acid (99.5–100.5%), ammonium acetate, acetic acid, and potassium ferrocyanide were purchased from Merck, Germany, while sorbic acid (99.0%) was purchased from China. Methanol (99.9%, HPLC-grade) was purchased from Thailand and zinc acetate (99%) was supplied from the UK. Deionized water was obtained from the Science, Technology, and Innovation National Laboratory (STINL). The analysis was carried out on Sky-ray Technology (USA) LC 310 HPLC which consists of a standard P100 high-pressure constant flow pump, UV detector, 7725i manual injector (Exformma Pronaos series chromatography) with 20 µL injection loop, and column (C18, 5 µm 6.4x 250 mm). The WS100 workstation software was used for the analysis and the Excel was used for data analysis.

HPLC conditions for analysis. The mobile phase was a mixture of methanol: ammonium acetate buffer (0.02 M, pH 6.75) in a ratio of 20:80 (v/v) that eluted in isocratic mode with a flow rate of 0.8 mL/min and column temperature of 30°C. The separated benzoic and sorbic acids were detected at a wavelength of 220 nm.

Standard solution preparation. A mixture of stock solution of 1000 mg/L benzoic acid and sorbic acid was prepared in a mixture of Methanol: DI water (60:40, v/v), then stored in an amber bottle at 4°C and kept away from light (ready for maximum usage of one month) (Ding et al., 2015). The interim mixture solution of 100 mg/L of benzoic acid and sorbic acid was prepared by diluting the stock solution with deionized water and stored in an amber bottle at 4°C. The working standard solutions required were prepared by series dilution of stock/or interim solution with deionized water.

Fish sauce samples preparation and analysis. 21 fish sauce samples that are available and most commonly used by Cambodian people were collected from Tuek Thlar, Pochentong, Tangkrosang, Makro, and KC markets in Phnom Penh. Those fish sauces were the products of Cambodia (11 brands), Vietnam (4 brands), and Thailand

(6 brands) which were labeled as C1-C11, V1-V4, and T1-T6, respectively. The fish sauce sample was prepared following these steps, i.e., 1-mL of fish sauce was diluted with 15 mL of DI water in a 25 mL centrifuge tube. After shaking the dilute sample, it was sonicated in a water bath at 50℃ for 20 minutes. The sample solution was cooled to room temperature, thereafter, 2 mL of 0.25 M potassium ferrocyanide and 2 mL of 0.5 M zinc acetate were added to the solution where a white precipitate was generated. The solution was then mixed using a vortex mixer and centrifuged at 5000 rpm for 5 minutes in order to collect the supernatant. Transfer the supernatant to a 25 mL volumetric flask and dilute with DI water to the mark. The mixture was then filtered through a 0.45 µm syringe filter before injecting into the HPLC. The prepared fish sauces were analyzed with HPLC using the optimized condition mentioned in *HPLC conditions for analysis*.

Results and Discussion

Chromatogram of benzoic and sorbic acid

To get a chromatogram with good separation and well-shaped peaks of benzoic and sorbic acids as well as a short analysis time, some parameters were tested before obtaining the optimal conditions. The standard mixture of 50 mg/L benzoic and sorbic acids was injected into the HPLC system under optimal conditions. The corresponding chromatogram obtained from the system is displayed in Fig. 2. As can be seen, both acids were well-separated and had well-defined peaks. The retention times of benzoic acid (BA) and sorbic acid (SA) were approximately 6.21 and 7.64 minutes, respectively. Another interference peak (negative peak) also appeared in the chromatogram; however, it is separated from benzoic and sorbic acids, leading to no influence on the analysis of acidic peaks.

HPLC-UV analytical performance

The analytical performance of HPLC which includes calibration curve, LOD, LOQ, and precision of the

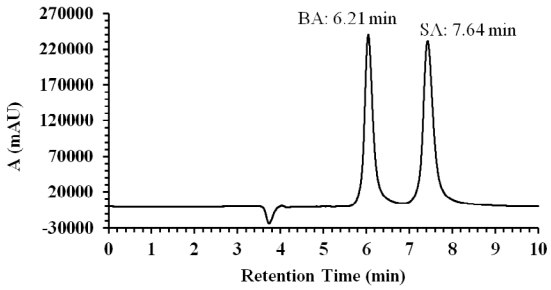

Fig. 2: Chromatogram of 50 mg/L of the standard mixture of benzoic and sorbic acids

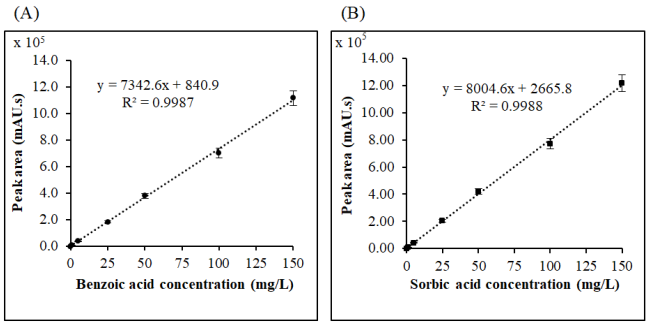

Fig. 3: The calibration curve for (A) benzoic acid and (B) sorbic acid, in the standard mixture with a concentration range from 0.05 to 150 mg/L

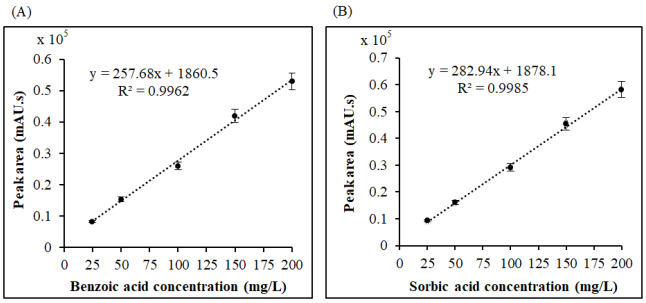

Fig. 4: Calibration curve of the method for (A) benzoic acid and (B) sorbic acid in the fish sauce spiked to get a concentration range of 25–200 mg/L

instrument was studied before method validation. The calibration curve was plotted between the average peak area values versus the standard concentration of benzoic acid and sorbic acid. The result revealed the linearity range from 0.05 to 150 mg/L for benzoic acid (Fig. 3A) and sorbic acid (Fig. 3B) with R^2 of 0.9987 and 0.9988, respectively. This indicated that benzoic and sorbic acids have a well-linear relation between their concentration and the peak area.

The LOD and LOQ were estimated from the standard mixture concentration of benzoic and sorbic acids which provided the ratio of the signal height and noise height about 3 and 10, respectively. The LOD and LOQ of each acid are presented in Table 1. The result showed that the instrument can detect benzoic and sorbic acid standards in very low concentrations compared to the limit set by Codex Alimentarius (1000 mg/L).

The precision of the HPLC system was evaluated based on the %RSD of the retention time and peak area of a 25 mg/L standard mixture of benzoic and sorbic acids as displayed in Table 2. When measuring the standard

Table 1: The LOD and LOQ of benzoic and sorbic acids in a standard mixture

Analyte	*LOD (mg/L)*	*LOQ (mg/L)*
Benzoic acid (BA)	0.025	0.090
Sorbic acid (SA)	0.025	0.090

Table 2: %RSD of the retention time and peak area of the 25 mg/L standard mixture of BA and SA (n = 5)

No.	BA		SA	
	RT (min)	*Peak area (mAU.s)*	*RT (min)*	*Peak area (mAU.s)*
1	6.17	1.87×10^5	7.61	2.09×10^5
2	6.23	1.85×10^5	7.66	2.05×10^5
3	6.22	1.84×10^5	7.65	2.09×10^5
4	6.19	1.85×10^5	7.64	2.06×10^5
5	6.16	1.85×10^5	7.58	2.05×10^5
Mean	6.19	1.85×10^5	7.63	2.06×10^5
SD	0.03	171	0.03	1515
%RSD	0.44	0.40	0.37	0.70

Table 3: The LOD and LOQ of BA and SA in the spiked fish sauce

Analyte	*LOD (mg/L)*	*LOQ (mg/L)*
Benzoic acid (BA)	10	30
Sorbic acid (SA)	10	30

multiple times, the obtained %RSD of retention time for BA and SA was 0.44 and 0.37%, respectively, which are lower than the acceptable value of 1%. Similarly, the %RSD of peak area was 0.40% for BA and 0.70% for SA, which are smaller than the acceptable value of 4%, indicating perfect precision of the instrument.

Method validation

Fish sauce samples were used to test the analytical applicability of the procedure, which included sample extraction and HPLC-UV, in terms of calibration curve, LOD, LOQ, precision, and accuracy. The calibration curves of the method for benzoic and sorbic acids were made by spiking the standard mixture of BA and SA into one brand of fish sauce (containing no both acids) following the procedure described in fish sauce samples preparation and analysis to get the concentration range from 25 to 200 mg/L. The spiked fish sauce of each concentration was prepared three times (n = 3) and was then analyzed by HPLC under the optimum conditions. Fig. 4A and Fig. 4B display the results. Between 25 and 200 mg/L, the concentration and peak area of the benzoic and sorbic acids were related linearly, with R^2 values of 0.9962 (BA) and 0.9985 (SA), which means the linearity was good.

The LOD and LOQ were given by the spiked concentration of the same fish sauce used in the method calibration curve above, which provided S/N ≥ 3 for the LOD and S/N ≥ 10 for the LOQ. The LOD of 10 mg/L was obtained for BA (S/N = 4.0) and SA (S/N = 3.3), and LOQ was the same, 30 mg/L, for BA (S/N = 11) and SA (S/N = 10). The results are summarized in Table 3. The LOD and LOQ are higher than those of a previous study but lower than

Table 4A: Precision of the method for benzoic acid at two concentration levels (n = 3)

Fish sauce	*Spike concentration (mg/L)*	*Concentration (mg/L)*	*RSD (%)*
C_1	25	22.8 ± 0.7	3.3
	200	186 ± 2.3	1.2
C_2	25	22.8 ± 0.8	3.7
	200	226 ± 1	0.6
C_3	25	23.7 ± 0.6	2.6
	200	177 ± 1.9	1.1
C_4	25	22 ± 0.5	2.2
	200	174 ± 0.8	3.6
C_5	25	23.8 ± 0.6	2.7
	200	168 ± 1	0.5
C_6	25	22 ± 0.7	2.9
	200	178 ± 2	1.1
C_7	25	22.2 ± 0.6	3.0
	200	187 ± 1	0.5
C_8	25	23.3 ± 0.6	2.5
	200	178 ± 1	0.4
C_9	25	24.1 ± 0.7	2.9
	200	179 ± 0.4	0.2
C_{10}	25	20.8 ± 0.8	3.7
	200	179 ± 1	0.4
C_{11}	25	21.4 ± 0.6	2.8
	200	165 ± 1	0.7
V_1	25	22 ± 0.6	2.7
	200	179 ± 1	0.5
V_2	25	22 ± 0.5	2.1
	200	163 ± 0.6	0.4
V_3	25	20.5 ± 0.2	1.2
	200	164 ± 0.8	0.5
V_4	25	24 ± 0.7	3.0
	200	162 ± 0.8	0.5
T_1	25	23.3 ± 0.8	3.5
	200	168 ± 0.4	0.3
T_2	25	22.8 ± 0.3	1.5
	200	186 ± 0.4	0.2
T_3	25	22.1 ± 0.5	2.3
	200	165 ± 1	0.7
T_4	25	21.8 ± 0.8	3.5
	200	167 ± 1	0.4
T_5	25	20.3 ± 0.7	3.2
	200	215 ± 1	0.3
T_6	25	184 ± 1	3.0
	200	162 ± 1	0.6

Table 4B: Precision of the method for sorbic acid at two concentration levels (n = 3)

Fish sauce	*Spike concentration (mg/L)*	*Concentration (mg/L)*	*RSD (%)*
C_1	25	22.3 ± 0.5	2.3
	200	191 ± 1	0.3
C_2	25	21 ± 0.7	3.4
	200	171 ± 2	1.3
C_3	25	22.4 ± 0.7	3.3
	200	166 ± 1.5	0.9
C_4	25	21.6 ± 0.5	3.6
	200	166 ± 0.8	0.5
C_5	25	23.8 ± 0.6	1.3
	200	167 ± 1	0.7
C_6	25	23 ± 0.9	3.8
	200	164 ± 1	0.7
C_7	25	22.9 ± 0.5	2.1
	200	161 ± 0.5	0.3
C_8	25	22.2 ± 0.5	2.2
	200	167 ± 0.8	0.5
C_9	25	22.8 ± 0.1	0.6
	200	168 ± 1	0.6
C_{10}	25	22.7 ± 0.6	2.4
	200	177 ± 0.9	0.5
C_{11}	25	21 ± 0.5	2.4
	200	164 ± 0.6	0.4
V_1	25	21.7 ± 0.7	3.4
	200	185 ± 0.7	0.4
V_2	25	21.8 ± 0.8	3.9
	200	171 ± 0.7	0.4
V_3	25	20.8 ± 0.6	2.7
	200	171 ± 0.4	0.2
V_4	25	22.4 ± 0.4	1.6
	200	161 ± 0.5	0.3
T_1	25	21.4 ± 0.6	2.9
	200	180 ± 0.8	0.5
T_2	25	23.9 ± 0.5	2.0
	200	161 ± 0.5	0.3
T_3	25	20.9 ± 0.5	2.4
	200	164 ± 0.5	0.3
T_4	25	23.4 ± 0.4	1.7
	200	177 ± 0.6	0.3
T_5	25	21.1 ± 0.7	3.3
	200	184 ± 0.9	0.5
T_6	25	23.2 ± 0.7	2.9
	200	170 ± 1	0.4

Table 5A: Recovery of the method for benzoic acid at two concentration levels (n = 3)

Fish sauce	*Spike concentration (mg/L)*	*Recovery (%)*
C_1	25	91.6 ± 3.3
	200	93 ± 1.2
C_2	25	91 ± 3.6
	200	113 ± 1
C_3	25	92.7 ± 2.6
	200	87.8 ± 1.1
C_4	25	86.6 ± 2.2
	200	87.2 ± 0.4
C_5	25	95.4 ± 2.6
	200	84.2 ± 0.5
C_6	25	86.5 ± 2.9
	200	88.8 ± 1.0
C_7	25	88.7 ± 2.9
	200	93.7 ± 0.5
C_8	25	93.3 ± 2.5
	200	89.0 ± 0.4
C_9	25	94.9 ± 2.9
	200	89.3 ± 0.2
C_{10}	25	83.2 ± 3.7
	200	89.3 ± 0.4
C_{11}	25	85.7 ± 2.7
	200	82.7 ± 0.7
V_1	25	88.9 ± 2.7
	200	89.7 ± 0.5
V_2	25	87.8 ± 2.1
	200	81.2 ± 0.4
V_3	25	81.8 ± 1.1
	200	82.0 ± 0.5
V_4	25	94.0 ± 3.0
	200	80.8± 0.5
T_1	25	93.1 ± 3.5
	200	84.1 ± 0.3
T_2	25	91.1 ± 1.5
	200	92.8 ± 0.2
T_3	25	88.6 ± 2.3
	200	82.6 ± 0.6
T_4	25	87.4 ± 3.5
	200	83.6 ± 0.4
T_5	25	80.7 ± 3.2
	200	107.3 ± 0.3
T_6	25	84.6 ± 3.1
	200	81.0 ± 0.5

Table 5B: Recovery of the method for sorbic acid at two concentration levels (n = 3)

Fish sauce	*Spike concentration (mg/L)*	*%Recovery*
C_1	25	89.4 ± 2.3
	200	95.7 ± 0.3
C_2	25	86.6 ± 3.4
	200	85.3 ± 1.3
C_3	25	89.4 ± 3.3
	200	83.0 ± 0.9
C_4	25	86.6 ± 3.6
	200	82.8 ± 0.5
C_5	25	95.4 ± 1.3
	200	83.5 ± 0.7
C_6	25	87.8 ± 3.7
	200	82.1 ± 0.7
C_7	25	91.4 ± 2.1
	200	80.7 ± 0.3
C_8	25	88.9 ± 2.2
	200	83.3 ± 0.5
C_9	25	91.4 ± 0.6
	200	84.2 ± 0.6
C_{10}	25	89.6 ± 2.4
	200	88.3 ± 0.5
C_{11}	25	83.6 ± 2.3
	200	82. ±2 0.4
V_1	25	86.8 ± 3.4
	200	92.3 ± 0.4
V_2	25	87.4 ± 3.8
	200	85.6 ± 0.4
V_3	25	83.3 ± 2.7
	200	85.5 ± 0.2
V_4	25	88.2 ± 1.6
	200	80.6 ± 0.3
T_1	25	85.5 ± 2.8
	200	90.0 ± 0.5
T_2	25	95.6 ± 2.0
	200	80.6 ± 0.3
T_3	25	83.7 ± 2.4
	200	81.8 ± 0.3
T_4	25	93.5 ± 1.7
	200	88.6 ± 0.3
T_5	25	84.5 ± 3.3
	200	91.8 ± 0.5
T_6	25	92.8 ± 2.8
	200	85.0 ± 0.4

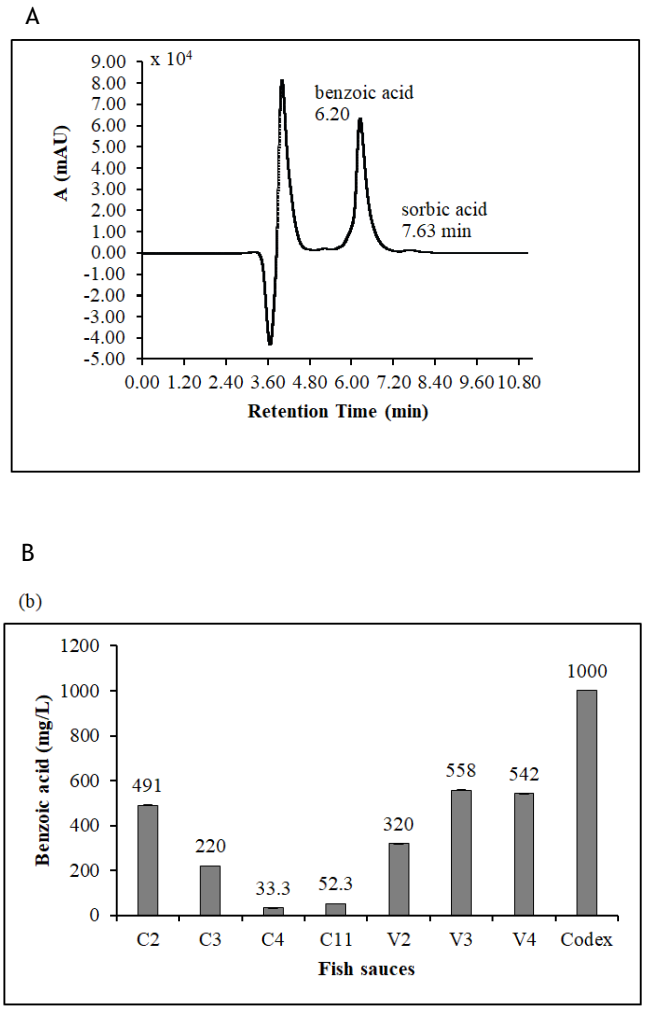

Fig. 5: **(A)** Chromatogram of fish sauce (V_4) and **(B)** Benzoic acid found in seven fish sauces, including Cambodian and Vietnamese brands and the permitted concentration of benzoic acid by Codex Alimentarius

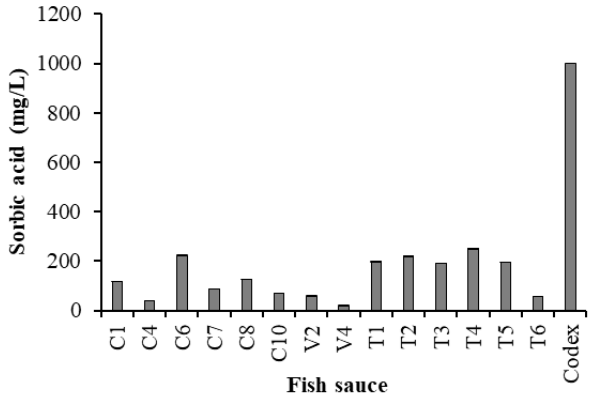

Fig. 6: Sorbic acid is found in 14 fish sauces, including Cambodian, Vietnamese, and Thai brands and the permitted concentration of sorbic acid by Codex Alimentarius

Table 6: The concentration of benzoic acid found in 21 fish sauces (n = 3)

Sample No.	*Fish sauce*	*Mean ± SD (mg/L)*
1	C_1	ND
2	C_2	491 ± 1
3	C_3	220 ± 1
4	C_4	33.3 ± 0.4
5	C_5	ND
6	C_6	ND
7	C_7	ND
8	C_8	ND
9	C_9	ND
10	C_{10}	ND
11	C_{11}	52.3 ± 0.8
12	V_1	ND
13	V_2	320 ± 2
14	V_3	558 ± 2
15	V_4	542 ± 1
16	T_1	ND
17	T_2	ND
18	T_3	ND
19	T_4	ND
20	T_5	ND
21	T_6	ND

ND = not detectable (< LOD)

Table 7: The concentration of sorbic acid found in 21 fish sauces (n = 3)

Sample No	*Fish sauce*	*Mean ± SD (mg/L)*
1	C_1	117 ± 1
2	C_2	ND
3	C_3	ND
4	C_4	37.7 ± 0.3
5	C_5	ND
6	C_6	223 ± 2
7	C_7	86.7 ± 0.3
8	C_8	125 ± 1
9	C_9	ND
10	C_{10}	69.7 ± 1
11	C_{11}	ND
12	V_1	ND
13	V_2	58.4 ± 0.8
14	V_3	ND
15	V_4	19.8 ± 0.7 (<LOQ)
16	T_1	199 ± 2
17	T_2	221 ± 2
18	T_3	190 ± 1
19	T_4	251 ± 2
20	T_5	195 ± 1
21	T_6	56.4 ± 1.1

ND = not detectable (< LOD)

the 1000 mg/L BA and SA levels allowed by the Codex Alimentarius (Codex, 1995). This implies that the method can effectively detect BA and SA in the actual sample. The precision of the method was studied by spiking the standard mixture of benzoic and sorbic acids to achieve two concentration levels, 25.0 and 200 mg/L, in all 21 fish sauces used for analysis. We similarly prepared the 21 fish sauces without spiking. The spiked fish sauces of each concentration and non-spiked fish sauces were prepared three times (n = 3) and were then analyzed by HPLC under the optimum conditions. The concentrations of BA and SA in the spiked and unspiked fish sauces were calculated using the linear equation obtained from the method as shown in Fig.4A and Fig. 4B. The results of the RSD for benzoic acid ranged from 0.2 to 3.7% (Table 4A) and for sorbic acid, it was 0.2 to 3.9% (Table 4B). The small value of RSD indicates the high precision of the analysis method.

The method's accuracy was conducted in the same way as described in the method precision study. The method yielded recoveries ranging from 80.8 to 113% for benzoic acid (Table 5A) and from 80.6 to 95.7% for sorbic acid (Table 5B). Those recoveries were in the acceptable range of 70 to 120% for the concentrations of 25.0 and 200 mg/L (Harnly, 2012), indicating high accuracy.

Determination of benzoic and sorbic acids in fish sauces

The BA and SA were first identified in the 21 fish sauces from the obtained chromatograms by comparing their retention time to the standard's retention time. If their retention times are matched with those of the standard, the BA or/and SA are presents in the sample. The peak areas of the BA and SA peaks were then used to calculate their concentration using the linear equation shown in Fig.4A and Fig. 4B. Fig. 5A displays the chromatogram of one fish sauce (V4), but it does not include the chromatograms of the other samples to avoid crowding. Table 6 provides the BA concentrations found in all 21 fish sauces.

In 11 fish sauces of the Cambodia brands, BA was found in 4 samples from 33.3 ± 0.4 to 491 ± 1 mg/L, while in the rest of the samples, the BA was not detectable. Three samples from four different Vietnamese brands ranged from 320 ± 2 to 558 ± 2 mg/L. The BA was not detectable in all six fish sauces of Thai brands. Therefore, 7 fish sauces, including Cambodian and Vietnamese brands, had BA levels between 33.3 ± 0.4 and 558 ± 2 mg/L. These levels are less than 1000 mg/L, which is the maximum level allowed by the Codex Alimentarius

(Fig. 5B). These types of fish sauces fall within the safe range of BA for human consumption.

Table 7 presents the concentrations of SA found in all fish sauces. As can be seen from the results, 6 samples of Cambodian brands were found to have SA in the range of 37.7 ± 0.3 to 223 ± 2 mg/L, while the 2 fish sauces of Vietnam brands contained SA from 19.8 ± 0.7 (<LOQ) to 58.4 ± 0.8 mg/L, and all 6 samples of Thai brands had the presence of SA in the range of 56.4 ± 1.1 to 251 ± 2 mg/L. The remaining 7 samples, 5 Cambodian brands and 2 Vietnamese brands, were not detectable. Therefore, 14 fish sauces, including Cambodian, Vietnamese, and Thai brands, contained SA in the range of 19.8 ± 0.7 (<LOQ) to 251 ± 2 mg/L, which is also lower than 1000 mg/L, the standard level permitted by Codex Almentarius, Fig. 6. So, these levels are still relatively safe for human consumption.

Conclusion

In this work, the HPLC-UV method is appropriate to estimate benzoic acid and sorbic acid in fish sauce simultaneously. Under the optimized condition of HPLC and simple extraction method of fish sauce using potassium ferrocyanide and zinc acetate, the method provided excellent linearity in the range of 25 to 200 mg/L with R^2 = 0.9962 (BA) and R^2 = 0.9985 (SA) with LOD = 10 mg/L and LOQ = 30 mg/L for both BA and SA. This method yielded high precision and recovery, as shown by an RSD from 0.2 to 3.9% and recovery from 80.6 to 113%, respectively, for both acids in 21 fish sauces at two spiked concentrations of 25 and 200 mg/L. Seven (Cambodian and Vietnamese brands) of the 21 fish sauces tested had BA levels between 33.3 ± 0.4 and 558 ± 2 mg/L. Another 14 fish sauces, including Cambodian, Vietnamese, and Thai brands, that make up 66.7% of the samples had SA levels between 19.8 ± 0.7 mg/L (<LOQ) and 251 ± 2 mg/L. Three fish sauces (C5, C9, and V1), comprising 14.3% of the samples, could not detect both BA and SA. The concentration of BA or SA in fish sauce remains below the limits set by the Codex Alimentarius, suggesting the safety of these fish sauces for human consumption. Our research remarks the analytical method which is reproducible and accurate for quantifying the benzoic and sorbic acid in fish sauce with the aid of sample preparation using potassium ferrocyanide and zinc acetate. The method is simple and can be utilized in extensive applications for food analysis. The results of BA and SA content contribute substantially as baseline data for future evaluation of preservative levels in fish sauces from different brands and areas. The differences in BA and SA concentration may be attributed to differing production methods, adherence to regulations, and changes in preservation practices. The absence of these in some samples points to the potential exploration of alternate preservation methods or tighter natural preservation regulations for some manufacturers. The monitoring of food preservatives is necessary for locally produced or imported fish sauces to ensure that the product meets the requirements of international safety standards. To raise awareness of consumers on food safety, the labeling of the preservative content must be restricted by the regulatory agency. Moreover, consumer education should be enhanced through public awareness campaigns on food preservatives and their health consequences. Having regionally harmonized regulations for food preservatives would also promote safe quality control and provide guidance to ensure uniformity of safety standards across multiple markets.

Acknowledgment

The authors acknowledge the Science, Technology, and Innovation National Laboratory (STINL) for supplying the facilities and requisite chemicals for the research. She conveys her appreciation to the Swedish International Development Cooperation Agency (SIDA), via Sweden and the Royal University of Phnom Penh (RUPP)'s Pilot Research Cooperation Program (Sida Contribution No. 11599), for the financial assistance granted to her supervisor for oversight during the research.

Declaration of Competing Interest

The author has no competing interests to declare.

Credit Authorship Contribution Statement

Mrs. TOM Sakmay designed and executed the experiment, analyzed the data, and composed the manuscript draft. Ms. PHAL Sereilakhena supervised Sakmay during her experimentation, data analysis, and manuscript composition and also revised all figures and the text draft.

Data Availability Statement

The author collected raw data. The data supporting the findings of this investigation are obtainable upon request from the author. The data are inaccessible to the public owing to privacy or ethical constraints.

Funding Declaration

No funding was received to assist with the preparation of this manuscript.

Author's Biography

Mrs. TOM Sakmay obtained a Master of Science in Chemistry from the Royal University of Phnom Penh (RUPP) in 2023. She is presently employed in the Science, Technology and Innovation National Laboratory (STINL) under the Ministry of Industry, Science, Technology, and

Innovation (MISTI). In 2015, she served as an analyst at the Food Chemical Laboratory (FCL) in the Ministry of Industry and Handicraft, which was subsequently renamed the Ministry of Industry, Science, Technology & Innovation in 2020. She participated in multiple training programs, including installation and maintenance of chemical testing utilizing Gas Chromatography in Korea in 2017, high-quality development of cross-border e-commerce in food circulation for belt and road countries in China in 2022, and advanced techniques such as high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), ion chromatography (IC), and spectrometry at the Science, Technology and Innovation National Laboratory (STINL) in Cambodia.

Ms. PHAL Sereilakhena received her doctorate in analytical chemistry from Umea University in Umea, Sweden, in 2019. She has served as a lecturer in the Chemistry Department at the Faculty of Science, Royal University of Phnom Penh (RUPP) since 2011. In 2015, she participated in the Linnaeus-Palme program as an exchange lecturer at Umea University for one month. Furthermore, from 2022 to 2024, she worked as the Chemistry Sub-Programme Coordinator for the SIDA-funded Sweden-RUPP bilateral initiative. She oversees the full-time Master of Science in Chemistry program at the Graduate School of Science (GSS), RUPP. She was elevated to assistant professor in 2023. She serves as the head of research for the Executive Committees of the Cambodian Chemical Society (CCS) and is a member of ANFEC in Cambodia with assistance from ISP. In 2023, she obtained a staff mobility grant from the Erasmus+ ICM program. Her primary scientific interests encompass sample preparation techniques, development of electrochemical and colorimetric sensors.

References

Amit, S. K., Uddin, M. M., Rahman, R., Islam, S. M. R., & Khan, M. S. (2017). A review on mechanisms and commercial aspects of food preservation and processing. *Agriculture & Food Security*, *6*(1). https://doi.org/10.1186/s40066-017-0130-8

Ammen, E. W., & Al-Salhi, R. (2017). An application of gas chromatography-mass spectrometry (GC-MS) fast automated scan/SIM type (FASST) in determining the preservative levels in foods. *American Journal of Analytical Chemistry*, *08*(05), 307-316. https://doi.org/10.4236/ajac.2017.85023

Burana-osot, J., ; Arunsingkharat, L. N., Matsarin; Naungnamjai, Supapan , & and Saetun, T. (2014). Validation of a HPLC method for the determination of benzoic acid and sorbic acid in noodles. *Chiang Mai J. Sci*, *41*(2), 370-382. https://epg.science.cmu.ac.th/ejournal/journal-detail.php?id=4822

Chuon, M. R., Shiomoto, M., Koyanagi, T., Sasaki, T., Michihata, T., Chan, S., Mao, S., & Enomoto, T. (2014). Microbial and chemical properties of Cambodian traditional fermented fish products. *J Sci Food Agric*, *94*(6), 1124-1131. https://doi.org/10.1002/jsfa.6379

Codex, A. (1995). *Codex Alimentarius (international food standard): General standard for food additive CXS 192-1995*. WHO & FAO. https://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/gsfa/en/

Ding, M., Peng, J., Ma, S., & Zhang, Y. (2015). An environment-friendly procedure for the high performance liquid chromatography determination of benzoic acid and sorbic acid in soy sauce. *Food Chemistry*, *183*, 26-29. https://doi.org/https://doi.org/10.1016/j.foodchem.2015.03.025

Golge, O., Hepsağ, F., & Kabak, B. (2015). Dietary intake of sorbic and benzoic acids from tomato ketchup for adults and children in Turkey. *Journal für Verbraucherschutz und Lebensmittelsicherheit*, *10*, 341-347.

Harnly, M. (2012). AOAC international guidelines for validation of botanical identification methods, Botanical Guidelines: Journal of AOAC International. *95*. https://doi.org/org/10.5740/jaoacint.11-266)

Javanmardi, F., Nemati, M., Ansarin, M., & Arefhosseini, S. R. (2015). Benzoic and sorbic acid in soft drink, milk, ketchup sauce and bread by dispersive liquid–liquid microextraction coupled with HPLC. *Food Additives & Contaminants: Part B*, *8*(1), 32-39. https://doi.org/10.1080/19393210.2014.955534

Kim, D. B., Jang, G. J., Yoo, M., Lee, G., Yun, S. S., Lim, H. S., Kim, M., & Lee, S. (2018). Sorbic, benzoic and propionic acids in fishery products: a survey of the South Korean market. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, *35*(6), 1071-1077. https://doi.org/10.1080/19440049.2018.1447692

Mustafa, A., Ayub, R., Irfan, S., Iftikhar, B., & Inamullah, M. (2021). Determination of food preservatives (benzoic and sorbic acids) in bakery products of district Peshawar, Pakistan. *Journal of Medical Sciences*, *29*(03). https://doi.org/10.52764/jms.21.29.3.5

Özdemir, A., Şanlı, S., Sardoğan, B., & Sardoğan, S. (2020). Determination of sorbic acid in cheese samples by rapid HPLC-DAD method. *Int J Anal Chem*, *2020*, 6049028. https://doi.org/10.1155/2020/6049028

Saad, B., Bari, M. F., Saleh, M. I., Ahmad, K., & Talib, M. K. (2005). Simultaneous determination of preservatives (benzoic acid, sorbic acid, methylparaben and propylparaben) in foodstuffs using high-performance liquid chromatography. *J Chromatogr A*, *1073*(1-2), 393-397. https://doi.org/10.1016/j.chroma.2004.10.105

Sirhan, A. Y. (2018). Optimization and validation of an HPLC-UV method for determination of benzoic acid and sorbic acid in yogurt and dried-yogurt poroducts using a design of experiment. *Indonesian Journal of Chemistry*, *18*(3), 522 - 530. https://doi.org/10.22146/ijc.27675

Theary, C., Panagides, D., Laillou, A., Vonthanak, S., Kanarath, C., Chhorvann, C., Sambath, P., Sowath, S., & Moench-Pfanner, R. (2013). Fish Sauce, Soy Sauce, and Vegetable Oil Fortification in Cambodia: Where do we Stand to Date? *Food and Nutrition Bulletin*, *34*(2_suppl1), S62-S71. https://doi.org/10.1177/15648265130342s108

Thongthai, C., & Gildberg, A. (2005). Asian fish sauce as a source of nutrition. *Asian functional foods*, 215-265.

Timofeeva, I., Kanashina, D., Stepanova, K., & Bulatov, A. (2019). A simple and highly-available microextraction of benzoic and sorbic acids in beverages and soy sauce samples for high performance liquid chromatography with ultraviolet detection. *Journal of Chromatography A*, *1588*, 1-7. https://doi.org/https://doi.org/10.1016/j.chroma.2018.12.030

Tungkijanansin, N., Alahmad, W., Nhujak, T., & Varanusupakul, P. (2020). Simultaneous determination of benzoic acid, sorbic acid, and propionic acid in fermented food by headspace solid-phase microextraction followed by GC-FID. *Food Chem*, *329*, 127161. https://doi.org/10.1016/j.foodchem.2020.127161

Wen, Y., Wang, Y., & Feng, Y.-Q. (2007). A simple and rapid method for simultaneous determination of benzoic and sorbic acids in food using in-tube solid-phase microextraction coupled with high-performance liquid chromatography. 388:1779–1787. https://doi.org/10.1007/s00216-007-1395-8

Xu, C., Liu, J., Feng, C., Lv, H., Lv, S., Ge, D., & Zhu, K. (2019). Investigation of benzoic acid and sorbic acid in snack foods in Jilin province, China. *International Journal of Food Properties*, *22*(1), 670-677. https://doi.org/10.1080/10942912.2019.1599011

Zeuthen, P., & Bøgh-Sørensen, L. (2003). *Food preservation techniques*. CRC Press ; Woodhead. http://www.crcnetbase.com/isbn/9780849317576 wv