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Optimized HPLC-UV Methodology for the Simultaneous Quantification of Multiple Preservatives in Jordanian Yogurt Products

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ABSTRACT

A method based on high-performance liquid chromatography with a UV detector (HPLC-UV) was developed for the simultaneous determination of sorbic acid, benzoic acid, and natamycin in yogurt. The method does not require time-consuming, labor-intensive pre-treatment processes or complicated procedures. Using a C18 150 mm × 4.6 mm, 3.0 µm column (Roc) at 25 °C, the target analytes were separated within 5 minutes with high sensitivity and selectivity. The mobile phase consisted of trifluoroacetic acid (0.1%) in water containing 100 mM sodium acetate, trifluoroacetic acid (0.1%) in acetonitrile, and trifluoroacetic acid (0.1%) in tetrahydrofuran, in a ratio of 70:20:10 (v/v). Using this mobile phase as an extraction mixture, recoveries ranged from 83.0% to 110.2% at spike levels between 2.5 µg/kg and 80.0 µg/kg. The relative standard deviations (RSDs) for these recoveries were below 10%. Intra-day precision and inter-day precision varied from 5.3% to 6.7% and 7.6% to 9.2%, respectively. Additionally, the limits of detection (LOD) were between 0.24 and 0.61 mg/L, and the limits of quantification (LOQ) ranged from 0.80 to 2.0 mg/L for sorbic acid, benzoic acid, and natamycin. Principal component analysis revealed that yogurt type had the greatest positive influence on preservative concentration, while the weight or volume of the yogurt package had the greatest negative influence.

Keywords: HPLC, Benzoic acid, Sorbic Acid, Natamycin, Preservatives, Principal component analysis (PCA).

INTRODUCTION

Yogurt is a nutritious food made from milk (lactose) that has undergone bacterial fermentation (1) and has been acidified with viable and well-defined bacteria (2). The bacteria used in the yogurt industry are generally referred to as "yogurt cultures" (3). Lactic acid bacteria ferment lactose, a disaccharide made up of galactose and glucose that accounts for about 4–5% of milk by weight (4), into lactic acid. This lactic acid interacts with milk proteins to produce yogurt, giving it its characteristic texture and tart flavor. Yogurt typically contains the bacterial cultures *Lactobacillus bulgaricus*, *Lactobacillus delbrueckii* subsp., and *Streptococcus thermophilus* (5). Additionally, other lactobacilli and bifidobacteria are sometimes added during or after the yogurt culturing process (6).

In yogurt production, milk is heated to around 85°C to denature the proteins and prevent curd formation. Afterward, the milk is cooled to approximately 45°C (7, 8). The bacterial culture is then mixed in and maintained at 45°C for four to twelve hours to allow fermentation to occur (9).

Yogurt produced following good manufacturing practices is expected to have a shelf life of about three

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days, with less than one yeast cell per gram during transportation and storage under refrigerated conditions in retail outlets (10). However, yogurt has been reported to contain yeast and mold contaminants, with yeast counts ranging from 2.39 to 5.39 log colony-forming units (CFU) per gram (11, 12). High yeast counts in milk have been attributed to factors such as inadequate heat treatment, contamination of utensils and air, the use of starter cultures prepared from previous-day milk, and temperature abuse during treatment (13, 14).

To improve yogurt consistency and extend its shelf life, additives are commonly used to prevent microbial attacks. Preservatives, technically speaking, are chemicals that inhibit microorganisms, preventing food from fermenting and spoiling without posing harm to the consumer (15). Food preservatives such as benzoic acid, sorbic acid, propionates, and dimethyl dicarbonate are used as antimicrobials, while ascorbic acid and butylated hydroxyanisole serve as antioxidants. Antibiotics like oxytetracycline, nisin, and natamycin are also employed in some cases (16).

Commonly used additives in the yogurt industry include benzoic acid, sorbic acid, and their metal derivatives (17). The presence of these additives in yogurt poses potential health risks to consumers. Therefore, permissible levels have been established to prevent the misuse of preservatives. The Joint FAO/WHO Expert Committee on Food Additives set an allowable daily intake of 5 mg/kg/day for benzoic acid and 25 mg/kg/day for sorbic acid (18, 19).

Sample preparation is often regarded as a crucial stage in the analysis of preservatives since it heavily depends on the chemical and physical properties of the contaminants. Food products with high levels of fat and protein, such as yogurt, require a complex, multi-step treatment process. Most methods for extracting these preservatives involve cumbersome, laborious, and time-consuming pre-treatment techniques, including the use of primary extraction solvents or a mixture of organic solvents (20, 21).

Developing an extraction method requires a fundamental understanding of extraction principles, including the transfer of target analytes from the sample matrix to the extracting phase (22). Factors such as selectivity, speed, and sample throughput vary depending on the extraction approach used (23). Once extracted, preservatives are detected and quantified using various analytical methods, such as gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) coupled with ultraviolet-visible (UV-Vis) detection, and liquid chromatography coupled with mass spectrometry (LC-MS) (18, 24, 25).

In this study, we employed a simple solvent extraction followed by HPLC-UV analysis for the detection of sorbic acid, benzoic acid, and natamycin in yogurt samples.

EXPERIMENTAL

Reagents and materials

Standard solutions of benzoic acid (99.6%) and sorbic acid (99.0%) were purchased from Acros Organics (Switzerland). A standard solution of natamycin (100%) was obtained from the U.S. Pharmacopeia (Rockville, MD, USA). Acetic acid (99.8%) was sourced from Fluka (Switzerland), while sodium acetate anhydrous (extra pure, SLR) was acquired from Fisher Chemical™ (UK). HPLC-grade methanol was provided by Labscan (Ireland), and a 0.45 µm disposable nylon syringe filter (25 mm) was purchased from Shimadzu (Japan).

HPLC analysis

HPLC analysis was performed using a Shimadzu LC-2030C 3D Plus system (Kyoto, Japan). The system included a four-solvent low-pressure gradient pump, degasser, autosampler with a 200 µL sample loop, column oven, and photodiode array detector, all controlled by LabSolution software (version 5.90, Shimadzu, Japan). Separation was carried out using a Roc C18, 3.0 µm, 4.6 x 150 mm chromatographic column from RESTEK (Pennsylvania, USA).

Sample extracts were analyzed using HPLC with a mobile phase consisting of three solutions:

- **Solution A**: 0.1% trifluoroacetic acid in water containing 100 mM sodium acetate
- **Solution B**: 0.1% trifluoroacetic acid in acetonitrile
- **Solution C**: 0.1% trifluoroacetic acid in tetrahydrofuran

The mobile phase was prepared by mixing the solutions in a 70:20:10 ratio (v/v).

Sample preparation

Target analytes were extracted from the yogurt samples using a solid-liquid extraction technique. Briefly, well-homogenized samples (5 g) were weighed into a 250 mL conical flask containing 50 mL of the mobile phase, comprising solutions A, B, and C in a 70:20:10 ratio (v/v). The mixture was stirred at high speed for 3 minutes, then filtered through filter paper, followed by filtration through a 0.45 µm disposable membrane filter prior to HPLC-UV analysis.

The target analytes were quantified using the external standard calibration method at five concentration levels ranging from 2.5 to 80 mg/L in the mobile phase. Both the standard solutions and samples were analyzed in triplicate, and calibration curves for each analyte were constructed by plotting the average chromatographic peak area against the standard concentration (26).

RESULTS AND DISCUSSION

Optimization of HPLC conditions

The peaks in the chromatograms obtained from HPLC-UV were identified and confirmed by matching their spectra with those of the standard solutions. The UV spectrum of natamycin displayed three main absorption peaks between 290 and 320 nm, with 302 nm selected for the identification and detection of natamycin (27). For benzoic acid, the UV spectrum showed two broad bands around 190 nm and 230 nm. To avoid the UV absorbance cut-off of the selected mobile phase, the peak at 227 nm, which gave higher absorption than the 190 nm peak, was chosen for the detection and quantification of benzoic acid. The maximum UV absorption for sorbic acid was found between 252 and 256 nm, with 254 nm used for its detection (28).

A 40 mg/L mixture of standard solutions of benzoic acid, natamycin, and sorbic acid was used to optimize the chromatographic conditions in a single run. The analytical column was a Brownlee Analytical 5 µm C18, 250 mm × 4.6 mm, operated at 25°C. The mobile phase consisted of an isocratic acetate buffer (pH 5.6) and methanol mixture in a 60:40 v/v % ratio (29). Preliminary investigations focused on the chromatographic retention of the target analytes, examining the effect of mobile phase composition and polarity. The influence of C8 and C18 columns with varying particle sizes and lengths, at temperatures ranging from 15°C to 30°C, was also explored.

In this experiment, different combinations of trifluoroacetic acid in water (containing 100 mM sodium acetate), acetonitrile, and tetrahydrofuran were tested in different ratios—70:20:10, 65:25:10, and 60:20:20 (v/v%)—for the HPLC mobile phase to optimize the resolution of sorbic acid, benzoic acid, and natamycin and improve sensitivity. When the mobile phase solutions A, B, and C were combined in a 60:20:20 (v/v%) ratio, overlapping peaks were observed between benzoic acid and natamycin. Additionally, using solutions A, B, and C in a 65:25:10 (v/v%) ratio resulted in poorly resolved peaks (resolution Rs ≈ 1.1) with shorter retention times. The mobile phase composed of solutions A, B, and C in a 70:20:10 (v/v%) ratio provided optimal separation between the benzoic acid and sorbic acid peaks (Rs > 1.5) with short retention times under 6 minutes and was selected for subsequent analysis.

To determine the optimum column temperature, four different temperatures (15°C, 20°C, 25°C, and 30°C) were tested, using the resolution factor and peak area as key criteria. The optimal temperature was found to be 25°C, which was subsequently used for further analysis. Under these conditions, effective separation of the target analytes was achieved, with retention times for all analytes within 5 minutes. The retention times were 2.68 minutes for

sorbic acid, 3.05 minutes for benzoic acid, and 4.95 minutes for natamycin (Fig. 1).

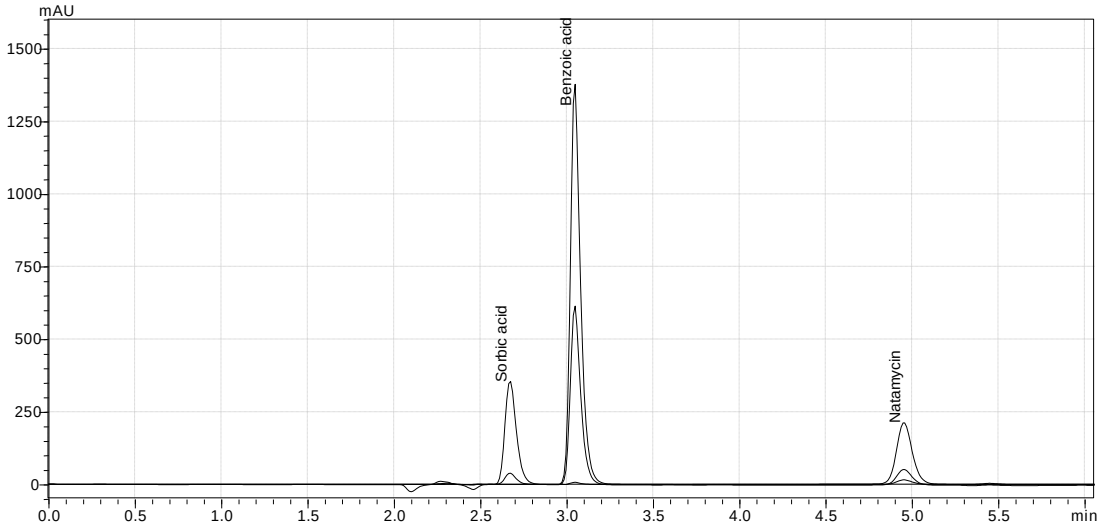


Figure 1: HPLC chromatogram of preservative standard solutions

Optimization extraction conditions

The extraction step is often considered the bottleneck of analytical methodologies and is recognized as the most critical step in sample preparation and chromatographic analysis (30, 31). The extraction technique employed must be both effective and efficient, with high sample throughput. Several methods have been used for the extraction and pre-concentration of preservatives from complex sample matrices, but these often involve multiple steps, are time-consuming, expensive, and labor-intensive (30).

When the mobile phase was used as the extraction solvent, the peak area of sorbic acid increased 1000-fold. However, there was a 54% reduction in the peak area of benzoic acid, while no significant change was observed in the peak area of natamycin (Table 1).

Table 1: Peak area of preservatives in standards mixtures extracted using different procedures method.

Preservative standards mixture	Extraction Methods	
	Mobile Phase (Peak Area)	Extraction Solvent (Peak Area)
Sorbic Acid	4401440	4525
Benzoic Acid	9838464	17265845
Natamycin	1228470	1182222

Validation of analytical methodology

The method was validated in terms of linearity, accuracy, intra- and inter-day precision, limits of quantification (LOQ), and limits of detection (LOD). Linearity was assessed using standard mixtures of the target analytes, with concentration ranges of 3.12–50.0 mg/L for sorbic acid and benzoic acid, and 2.50–40 mg/L for natamycin. As shown in Table 2, the calibration curves demonstrated good linearity, with correlation coefficients greater than 0.99, indicating a strong linear relationship between the concentration of the target analytes and the chromatographic peak response.

Table 2: Linearity range, Equation, r^2 value, LOD and LOQ of the target analytes

Preservative	Linearity Range (mg/L)	Equation	r^2	LOD (mg/L)	LOQ (mg/L)
Sorbic Acid	3.12 – 50.0	Y = (-87858) + (65255) X	0.9999	0.24	0.80
Benzoic Acid	3.12 – 50.0	Y = (-201579) + (142279) X	0.9998	0.39	1.3
Natamycin	2.50 – 40.0	Y = (-126923) + (34612) X	0.9975	0.61	2.0

The limits of detection (LOD) and quantification (LOQ) were estimated using signal-to-noise ratios of 3 and 10, respectively, from preservative-free samples (Table 2). The LODs were 0.66 mg/L for sorbic acid, 0.51 mg/L for benzoic acid, and 0.01 mg/L for natamycin.

The accuracy of the method was evaluated by calculating the average recoveries of the target analytes from diluted yogurt samples spiked with three different concentrations (5, 10, and 20 mg/L) of the preservative standard mix, each analyzed in triplicate. The recovery was calculated using Equation 1 (32):

$$\text{Recovery (\%)} = \frac{\text{Recovered Amount (mg/L)}}{\text{Added Amount (mg/L)}} \times 100 \quad (1)$$

As shown in Table 3, recoveries ranged from 83.0% to 112.8%, with relative standard deviations (RSD) between 1.3% and 9.6%. Sorbic acid exhibited the highest recoveries (97.7%–112.8%), followed by benzoic acid (89.9%–96.3%), while natamycin showed the lowest recoveries (84.9%–85.7%).

Table 3: Average recoveries and Relative standard deviation (RSD) of the target analytes

Preservative	Spiking Level (mg/L)	Yogurt	Diluted Yogurt
		Mean of Recovery (%) ± RSD (%)	
Sorbic Acid	5	112.8±6.7	83.0±7.7
	10	97.9±6.0	88.6±8.8
	20	97.7±5.3	108.1±4.9
Benzoic Acid	5	91.4±1.3	86.0±7.4
	10	89.9±8.3	110.2±5.0
	20	96.3±5.4	96.5±6.3
	5	84.9±7.4	86.0±7.4
Natamycin	10	85.7±9.3	110.2±5.0
	20	85.4±6.7	96.5±6.3

In this study, five extractions were performed in a single day by spiking preservative-free samples with 20 mg/L of the target analytes to determine intra-day precision. Inter-day precision was assessed by performing five extractions per day over three days. The calculations and results for intra-day and inter-day precision are presented in Table 4.

The intra-day precision (n = 5) ranged from 5.3% to 6.7%, while the inter-day precision ranged from 7.6% to 8.0%. The figures of merit for the analytical methodology obtained in this study were satisfactory and comply with the SANTE 11312/2021 guidelines (33).

Table 4: The intra-day precision and inter-day precision of the developed method

Preservative	Spiking Level (mg/L)	Intra-Day Precision (n = 5)[a]	Inter-Day Precision (n = 15)[a]
Sorbic Acid	20	5.3	7.6
Benzoic Acid	20	5.4	8.0
Natamycin	20	6.7	9.2

Analysis of samples

The developed method was applied to analyze local and imported yogurt samples purchased from Jordanian markets. A total of 120 dried yogurt samples were analyzed, consisting of 60 Jameed yogurt samples (locally produced in Jordan and some imported from Hungary, Turkey, Syria, and Egypt) and 20 liquid Jameed yogurt samples from Jordan. Benzoic acid was detected at varying concentrations (13.1–97.3 mg/L) in 19 samples of Jameed (4 from Jordan, 5 from Syria, and 9 from Egypt) and in 1 sample of liquid Jameed yogurt. Natamycin was found in 13 samples of dried yogurt and 1 sample of liquid Jameed yogurt. Sorbic acid was detected in 14 separate samples of dried yogurt from Jordan at concentrations ranging from 1.61–22.72 mg/L and 5.87–383.69 mg/L. No preservatives were detected in the remaining 151 yogurt samples obtained from Jordan, Hungary, and Turkey. Figure 2 displays an HPLC chromatogram of a dried yogurt sample with 30 mg/kg natamycin. The detection of benzoic acid in the yogurt correlates with the study by Mazdeh and colleagues, who detected benzoate and sorbate in concentrations ranging from 2.08–58.19 mg/kg and 3.81–246.60 mg/kg, respectively, in yogurt samples obtained in Iran (18).

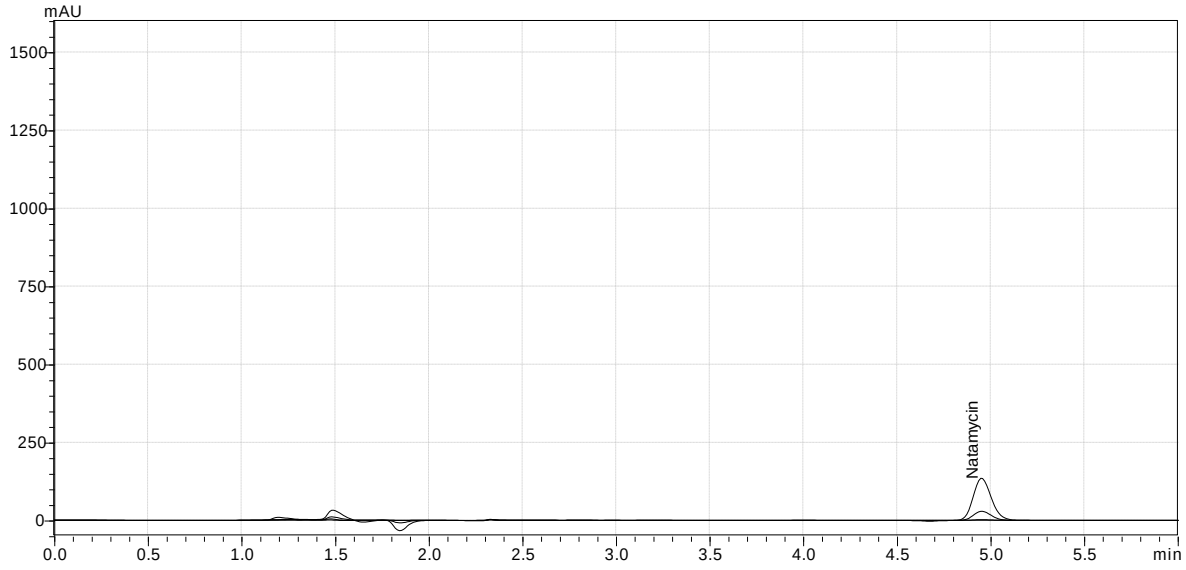


Figure 2: HPLC chromatogram of a yogurt sample containing 30 ppm natamycin.

Statistical analysis

Samples were analyzed randomly after being coded and evaluated in triplicate. Principal component analysis (PCA) was used to quantitatively analyze the relationships between the types of yogurt and the concentrations of benzoic acid, sorbic acid, and natamycin (34). Two principal components with Eigenvalues greater than 1 explained 50.4% of the variation, as shown in the Scree plot (Figure 3).

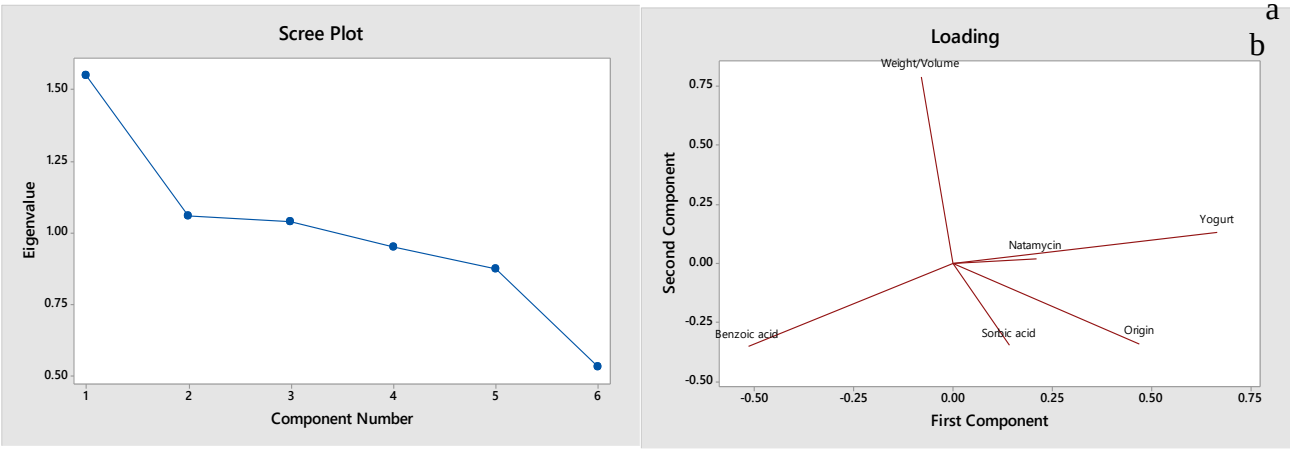


Figure 3: Scree plot and loading plot

Table 5: Eigenanalysis of the correlation matrix

Eigenvalue	1.5480	1.0580	1.0400	0.9502	0.8714	0.5324
Proportion	0.258	0.176	0.173	0.158	0.145	0.089
Cumulative	0.258	0.434	0.608	0.766	0.911	1.000
Eigenvectors						
Variable	PC1	PC2	PC3	PC4	PC5	PC6
Yogurt	0.665	0.130	–0.520	–0.132	–0.042	–0.720
Origin	0.469	–0.340	0.080	–0.602	0.291	0.459
Weight/Volume	–0.081	0.787	–0.214	–0.214	0.523	0.091
Benzoic acid	–0.517	–0.353	0.131	–0.359	0.447	–0.511
Sorbic acid	0.142	–0.350	–0.674	0.445	0.453	0.009
Natamycin	0.209	0.019	0.688	0.498	0.484	0.027

As shown in Table 5, the first three factors—type of yogurt, country of origin, and weight or volume of the yogurt—account for 60.80% of the variation in the concentration of preservatives in yogurt samples. This is also evident from the Scree plot (Figure 2a), which shows that these three components have Eigenvalues greater than 1, according to the Kaiser criterion (35). The loading plot (Figure 2b) indicates that the type of yogurt has the largest positive influence on the preservative content, followed by the country of origin. The weight or volume of the yogurt package has a weaker influence on preservative concentration. Additionally, it was observed that the

benzoic acid content has a significant negative impact on yogurt.

Table 5 also shows that the type of yogurt has a significant positive influence on the country of origin, and vice versa. The origin of the yogurt positively influences the type of yogurt, the weight or volume of the yogurt, and the concentrations of sorbic acid and natamycin. The weight or volume of the yogurt positively affects the origin and also the concentrations of sorbic acid and natamycin. Conversely, the concentration of benzoic acid is positively influenced by both the weight/volume of the yogurt and the concentration of sorbic acid. Additionally, the concentration of sorbic acid has a significant positive influence on the type of yogurt, the weight/volume of the yogurt, and the concentration of natamycin. The concentration of natamycin was found to have a large positive influence and strongly correlate with other variables. Thus, the preservative content of yogurt samples may be influenced by their source, as well as by storage and handling methods.

Comparison with previous studies

The developed method was compared with previous methods for analyzing preservatives in yogurt, as shown in Table 6, in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, and relative standard deviation.

Table 6: comparison of present study with previous studies

S/N	Method	Linearity (R^2) (mg/L)	LOD (mg/L)	LOQ (mg/L)	Recovery (%)	RSD (%)	Ref
1	Ultrasonic Extraction/HPLC	5.1 – 50 (0.9999)	10	50	91–105	8 – 8.3	(19)
2	Solvent extraction/HPLC	2 – 6 (n.r)	n.r	n.r	91.33–99.50	n.r	(36)
3	Solvent extraction/HPLC	0.01-0.8 (0.9991)	0.320	0.403	104	0.562	(37)
4	Solvent extraction/HPLC	5-40 (0.997)	0.326—.520	0.989-1.575	87.85–94.16	0.55–1.33	(18)
5	Solvent extraction/RP-HPLC	2.5-50 (0.997)	0.24–0.61	0.80–1.3	84.9–112.8	1.3-9.3	Present study

CONCLSION

The analysis of preservatives in yogurt has been facilitated by the development of a sample preparation method that is quick, easy, low-cost, effective, and efficient. The method was optimized for mobile phase composition, column type, column length, and particle size to enhance the sensitivity of HPLC-UV chromatography. It involves a single extraction step with no pre-treatment required. This method is recommended as an alternative for analyzing preservatives in food samples.

The method achieved good separation of target analytes with short retention times, using a C18 250 mm × 4.6 mm × 5 μm column at 25°C, with excellent selectivity and sensitivity. The method provided satisfactory figures of merit, including good linearity, accuracy (in terms of average recoveries), precision, and a low limit of detection, demonstrating its suitability for detecting preservatives in yogurt samples. The concentration of preservatives was found to be influenced separately and independently by the type of yogurt, origin, and weight/volume.

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طريقة HPLC-UV المحسنة لتقدير كمية المواد الحافظة المتعددة في منتجات الزبادي الأردنية في وقت واحد

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ملخص

تم تطوير طريقة تعتمد على تطبيق كروماتوغرافيا السائل عالية الأداء مع كاشف الأشعة فوق البنفسجية (HPLC-UV) لتحديد حمض السوربيك وحمض البنزويك والناتاميسين في الزبادي في وقت واحد. لم تتضمن الطريقة المطورة عمليات معالجة أولية تستغرق وقتًا طويلاً وتتطلب عمالة مكثفة أو إجراءً معقدًا. باستخدام عمود C18 150 مم 4.6 مم × 3.0 ميكرومتر (Roc) عند 25 درجة مئوية، تم فصل المحللات المستهدفة في 5 دقائق بحساسية وانتقائية عالية. يتكون الطور المتحرك من مزيج من حمض ثلاثي فلورو أسيتيك (0.1٪) في الماء يحتوي على 100 مليمول من أسيتات الصوديوم وحمض ثلاثي فلورو أسيتيك (0.1٪) في أسيتونتريل وحمض ثلاثي فلورو أسيتيك (0.1٪) في رباعي هيدروفوران (بنسبة 70:20:10، حجم / حجم). أدى استخدام الطور المتحرك كمزيج استخلاص إلى استردادات تتراوح من 83.0٪ إلى 110.2٪ عند مستويات الذروة التي تتراوح من 2.5 ميكروجرام / كجم إلى 80.0 ميكروجرام / كجم. كانت الانحرافات المعيارية النسبية (RSDs) المرتبطة بهذه الاستردادات أقل من 10٪. تراوحت النتائج الخاصة بالدقة داخل اليوم والدقة بين الأيام من 5.3٪ إلى 6.7٪ و 7.6٪ إلى 9.2٪ على التوالي. بالإضافة إلى ذلك، كانت حدود الكشف (LOD) بين 0.24 و 0.61 مجم / لتر وحدود التقدير (LOQ) بين 0.80 و 2.0 مجم / لتر لحامض السوربيك وحمض البنزويك والناتامايسين على التوالي. بناءً على تحليل المكونات الرئيسية، فإن نوع الزبادي له أكبر تأثير إيجابي على تركيز المواد الحافظة، في حين أن وزن أو حجم عبوة الزبادي له أكبر تأثير سلبي على تركيز المواد الحافظة.

الكلمات الدالة: HPLC، حمض البنزويك، حمض السوربيك، ناتامايسين، المواد الحافظة، تحليل المكونات الرئيسية (PCA).

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