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Development and validation of an RP-HPLC method for simultaneous determination of sorbic acid, benzoic acid, and natamycin in domestic yogurt in Jordan

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Abstract

A method for the simultaneous determination and analysis of sorbic acid, benzoic acid, and natamycin in yogurt products, based on the use of the high-performance liquid chromatography with a UV detector (HPLC–UV) has been developed. This method excludes the employment of complex procedures, time-consuming and labor-intensive pre-treatment processes. Separation of the sorbic acid, benzoic acid, and natamycin with higher selectivity and sensitivity, and within five minutes was performed by a C8 150 mm × 4.6 mm x 3 µm column (Hypersil™ BDS) at 20ºC. The mobile phase was a mixture 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 (65:25:10 (%, v/v)). Using the mobile phase as an extraction mixture gave recoveries in the range of 83.0-110.2% at spike levels of 2.5–80.0 µg/kg with relative standard deviations (RSDs) lower than 10%. The intra-day precision and inter-day precision results were in the range of 5.3–6.7% and 7.6–9.2%. Additionally, the limits of detection (LOD) were 0.24, 0.39, and 0.61 mg/L and the limits of quantification (LOQ) were 0.80, 1.3, and 2.0 mg/L for sorbic acid, benzoic acid, and natamycin, respectively.

Keywords: HPLC, Sorbic Acid, Benzoic Acid, Natamycin, Preservatives.

INTRODUCTION

Yogurt is a nutrient produced by bacterial fermentation of milk [1]. Bacteria used in the yogurt industry are known as "yogurt cultures". Fermentation of lactose, which is a disaccharide sugar composed of galactose and glucose and makes up around 2–8% of milk (by weight), by these bacteria produces lactic acid, which works on milk protein to give the yogurt its texture and characteristic tart flavor [1]. Yogurt contains the characteristic bacterial cultures Lactobacillus bulgaricus, delbrueckii subsp, and Streptococcus thermophilus. Besides, other lactobacilli and bifidobacteria are sometimes added during or after culturing of yogurt. Yogurt produced by heating milk, usually to around 85 °C (185 °F), to denature proteins to prevent form curds. Afterward, the milk is allowed to cool to around 45 °C (113 °F) [2]. Bacterial culture mixed and maintained at 45 ° C (113 ° F) for four to twelve hours to allow for fermentation [3].

When yogurt produced under good manufacturing practices besides kept under refrigeration during transportation and storage in retail outlets, yogurt should contain less than 1 yeast cell/g and have expected validity for 30 days [1,4]. Contamination with yeast and molds is common in yogurt production with yeast counts ranging between 2.39 and 5.39 log colony forming units (CFU)/g being reported [5].

Insufficient heat treatment of milk, contamination of utensils and air, the employment of previous-day milk as a starter and temperature abuse during treatment are responsible for high yeast counts in milk [6,7].

Therefore, additives are added to improve yogurt consistency, which extends the shelf life of foods and drinks by inhibiting microorganisms attack. Preservatives are technically chemicals that used to poison microorganisms as well to prevent the food added to it from fermentation and spoilage without causing any harmful impact to the person consuming the food. The roles of chemical preservatives to enhance food quality extend the shelf life of foods and enhance consumer acceptability [7]. The preservative is utilized widely in foods, and many researchers have observed it be powerful against microbial activities of yeasts and molds [8].

These chemical preservatives can be classified into three main types: Anti-microbial these include benzoic acid, sorbic acid, propionates, di-methyl pyrocarbonates, Antioxidant these include ascorbic acid, butylated hydroxyanisole, and antibiotic these include Oxytetracycline, ninsin, and natamycin.

The commonly used additives in yogurt manufacturing process in Jordan are Benzoic acid, or its Na, K, Ca salts, (E210–213), Sorbic acid or its Ca and K salts (E200–203), and Natamycin (also called pimaricin, E235). Using these additives are strictly prohibited as a preservative and its point of addition (during production) by current legislation, given the potential risks to consumers' health and safety [9].

– Benzoic acid, may be found in yogurt, since it may be a natural product of microbial metabolism [10]. Literature data [11] show that it may be found in yogurt because of technical

and microbiological reasons, even if it has not been added to yogurt during the production.

Benzoic acid is toxic and causes harmful effects such as metabolic acidosis, convulsions, and heberbenia have been detected in experimental animals and humans giving very high doses of benzoic acid. Some of the weak clastogenic activity was observed in vitro assays [7]. The evolution of allergic reactions to benzoates in humans, such as urticaria, non-immunological contact urticaria, and asthma, has been described in some studies of many benzoates that can be added to foods is carefully checked [12]. Codex Alimentarius, which is an international treaty dictating food safety standard, set limits on the amount of benzoic acid or sodium benzoate to 0.05 to 0.1 percent by volume.

- Sorbic acid has low toxicity and does not accumulate in the body, and illustrates the fact that the human body that is being catabolized to CO_2 and H_2O by beta-oxidation means that the human body rapidly metabolises sorbic acid by pathways similar to those of other fatty acids [13]. It was reported that a few cases of idiosyncratic intolerance in humans to sorbic acid.

– Natamycin is a small polyene macrolide antibiotic produced by actinomycetes Streptomyces natalensis [14,15]. The antifungal activities of natamycin act by binding to cell membrane sterols, especially ergosterol, the principal sterol in fungal membranes, thus making them leaky [16]. In the 2002 World Health Organization Food Additive Series: 48: Concluded, “The negative results in studies of acquired resistance indicate that selection of Natamycin-resistant fungi is not an issue” [17].

In general, sample preparation is regularly the most imperative part of the analysis of preservatives and depends mostly on the physical and chemical properties of the items that are contaminated with preservatives. Manufactured products with high fat and protein substance, for example, yogurt, require multi-steps treatment. Most techniques currently employed in extracting preservatives utilize muddled, tedious and labor intensive pre-treatment methodology incorporates the primary extraction with a solvent or a mixture of organic solvents, followed by precipitating of the fat and protein content by adding potassium hexacyanoferrate trihydrate solutions (6% w/v) in addition zinc acetate solutions [18]. Moreover, many of the published methods the analyte is prepared with multiple-steps steam distillation before treating using solid-phase extraction cartridge [19,20].

Analytical techniques for the analysis of sorbic acid, benzoic acid, and natamycin have been developed. The most common techniques for determination of sorbic acid, benzoic acid, and natamycin are high-performance liquid chromatography (HPLC) with a UV-Visible detector (UV) [21], gas chromatography (GC) [22], gas chromatography coupled with mass spectrometry (GC-MS) [23], and capillary electrophoresis [24]. Recently, liquid chromatography coupled with mass spectrometry (LC-MS) and liquid chromatography with tandem mass spectrometry (LC-MS/MS) [25] have been adopted.

This study aims to develop a simple and rapid method to determine simultaneously sorbic acid, benzoic acid, and natamycin in yogurt samples that can be used for quality control using simplified solvent extraction procedure followed by HPLC with a UV-Visible detector. The proposed method was in-house validated concerning linearity, accuracy, intra-day precision, inter-day precision, limit of detection (LOD), and limit of quantification (LOQ).

EXPERIMENTAL

Reagents and materials

Certified standard solutions of sorbic acid (99.0%) and benzoic acid (99.6%) were obtained from Acros Organics (Switzerland); certified standard solution of natamycin (100%) was obtained from U. S. Pharmacopeia (Rockville, MD, USA); acetic acid (99.8%), Fluka, Buchs, Switzerland; sodium acetate anhydrous Extra pure, SDFCL (India); Water (HPLC-grade) was purchased from VWR International (EC). Methanol (HPLC-grade) was purchased from Labscan (Dublin, Ireland). A nonsterile PTFE Syringe Filter with a disposable membrane filter (0.45 µm) was purchased from Whatman GmbH (Dassel, Germany).

HPLC analysis

The HPLC analysis was performed using a Thermo Scientific™ DIONEX UHPLC Plus Focused (UltiMate 3000) system (Thermo-Fisher, USA), the system consisting of a quaternary Series RS pump, a vacuum degasser, Thermo Scientific™ Dionex Ultimate 3000 Series TCC-3000RS column compartment, a Thermo Fisher Scientific™ Ultimate 3000 Series WPS-3000RS autosampler, and a variable UV wavelength detector and an autosampler.

The chromatographic separation was performed with Hypersil™ BDS, 3µm C8 150 mm × 4.6 mm chromatography column, which was purchased from Thermo Scientific™ (Shelton, USA).

The sample extracts were analyzed isocratically using a mixture of trifluoroacetic acid 0.1 % in water containing 100 mM sodium acetate (solution A), trifluoroacetic acid 0.1 % in acetonitrile (solution B), and trifluoroacetic acid 0.1 % in tetrahydrofuran (solution C) (65:25:10 (%, v/v)) as the mobile phase.

The wavelengths that have been applied for detection of sorbic acid, benzoic acid, and natamycin that gave maximum absorption were 260, 227, and 302 nm, respectively. The column was kept in a column oven at 20ºC at a flow rate of 1.0 mL/min to achieve the optimum resolution between benzoic acid and sorbic acid. The injection volume was maintained at 10 µL for both the sample and standard solutions.

Sample preparation

Samples were prepared similarly to the previous published benzoic acid and sorbic acid method with some modification [26]. The sorbic acid, benzoic acid, and natamycin were

extracted from the yogurt/dried yogurt samples using a solid-liquid extraction procedure. 5 g of well-homogenised samples were weighed into a 250 mL conical flask and dissolved in 50 mL of mobile phase (mixture of trifluoroacetic acid 0.1 % in water containing 100 mM sodium acetate (solution A), trifluoroacetic acid 0.1 % in acetonitrile (solution B), and trifluoroacetic acid 0.1 % in tetrahydrofuran (solution C) (65:25:10 (%, v/v)).). Then the mixtures were stirred for 3 min at high speed. After that, the mixture was filtered through a Whatman No.1 filter paper and passed through a 0.45 μm disposable membrane filter before HPLC analysis.

External standard calibrations were established for the quantification of sorbic acid, benzoic acid, and natamycin. The stock solution was prepared at 1000 mg/L for benzoic acid and sorbic acid at 500 mg/L for natamycin with methanol. Five concentration levels in the ranges of 2.5 –80 mg/L were prepared by diluting the stock solutions in the mobile phase (mixture of trifluoroacetic acid 0.1 % in water containing 100 mM sodium acetate (solution A), trifluoroacetic acid 0.1 % in acetonitrile (solution B), and trifluoroacetic acid 0.1 % in tetrahydrofuran (solution C) (65:25:10 (%, v/v)).

Under each of the conditions, samples as well standards were analyzed by duplicate injections. The linear equations and the correlation coefficients value of the determination were estimated by plotting a calibration curve between the peak area averages of the measured measurements versus the standard concentrations [27].

RESULTS AND DISCUSSION

Optimization of HPLC conditions

The peaks identification and purity were confirmed by matching their spectra (200–400 nm) with those of solutions of the standards. Peak determination and purity have been confirmed by comparing their spectra in the range of 200-400 nm with those standards of aqueous solutions.

The UV spectrum of natamycin indicates three main absorption peaks in the range of 290 to 320 nm. The wavelength that has been applied for detection that gave maximum absorption was at 302 nm, even though 304 nm is the wavelength that has been used typically to measure the antifungal [28]. The UV spectrum of benzoic acid consists of two broad bands located closely to 190 nm and 230 nm. The maxim peak at around 190 nm wavelength was not selected to avoid UV absorbance cutoff of selected mobile phase, additionally the peak at 227 nm gave higher absorption than the peak at 190 nm. Therefore, the peak at 227 was selected in quantifying benzoic acid in yogurt samples. On other hand, the UV spectrum of sorbic acid exhibits an absorption maximum at a wavelength of 252－256 nm, thus 254 nm was applied for detection of sorbic acid.

The optimization of the chromatographic conditions for separating sorbic acid, benzoic acid and natamycin in a single run was carried using a mix of preservative standards at 40 mg/L for sorbic and benzoic acids and 4 mg/L for natamycin. The analytical method used to separate sorbic acid, benzoic acid and natamycin based on conditions similar to the previously published benzoic acid and sorbic acid method with some modification.

The analytical column used on the previous published benzoic acid and sorbic acid method was a Brownlee Analytical, 5μm C18 250 mm × 4.6 mm, at 25 ºC and the mobile phase that used was isocratic 60:40 acetate buffer (pH 5.6) / methanol mixture [26]. Preliminary investigations involved the analysis of the chromatographic retention of the investigated analytes on mobile phase composition and polarity. Also, impact on the C8 and C18 columns with different particle size and length at a temperature ranging from 15 °C to 30 °C.

Through the preliminary study, factors that could deliver a stronger influence such as mobile phase composition were extracted for further analysis. The other factors such as flow rate (1 mL/ min), column temperature (25 °C), and column type and length were kept constant during the study. Preliminary experiments indicated that a mobile phase composed of trifluoroacetic acid 0.1 % in water, trifluoroacetic acid 0.1% in acetonitrile, and trifluoroacetic acid 0.1 % in tetrahydrofuran (60:20:20 (%, v/v)) lead to not resolved peaks, poor separation and very short retention time for that reason a 65% of trifluoroacetic acid 0.1 % in water was adopted. Preliminary experiments indicated that a mobile phase composed 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 (60:20:20 (%, v/v) lead to not resolved peaks, poor separation and very short retention time for that reason a 65% of trifluoroacetic acid 0.1 % in water was adopted. Subsequently different combinations 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 such as 65:15:20, 65:20:15, 65:25:10, 65:30:5, and 65:35:0 (%, v/v) were tested as the mobile phase in order to optimize the resolution of the sorbic acid, benzoic acid and natamycin and to enhance the sensitivity.

Overlapped peaks were observed between benzoic acid and natamycin when using the first mobile phase composition (65:15:20 (%, v/v)) as well as between benzoic acid and sorbic acid when using the last mobile phase composition (65:35:0 (%, v/v)). On the other hand, not well-resolved peaks (resolution (Rs) < 1.5) broad peaks and shorter retention times were produced using the second and fourth mobile phase systems. In contrast, the third mobile phase 65:25:10 (%, v/v) offered an optimum separation between the between benzoic acid and sorbic acid peaks (Rs > 2.8) with reasonable and short retention times with 10 minutes so that it was adopted for later investigation.

The effect of different temperatures (15°C, 20°C, 25°C, and 30°C) was studied to find out the optimum separation, using the resolution factor; and the peaks area are as basic criteria. The temperature of 20°C was adopted as it gave the best results. These analytical conditions led to an effective separation of the targeted preservatives for all products analyzed within 10 min. The average retention time was 7.0 min for sorbic acid and 7.8 min for benzoic acid and 9.4 min for natamycin (Fig. 1).

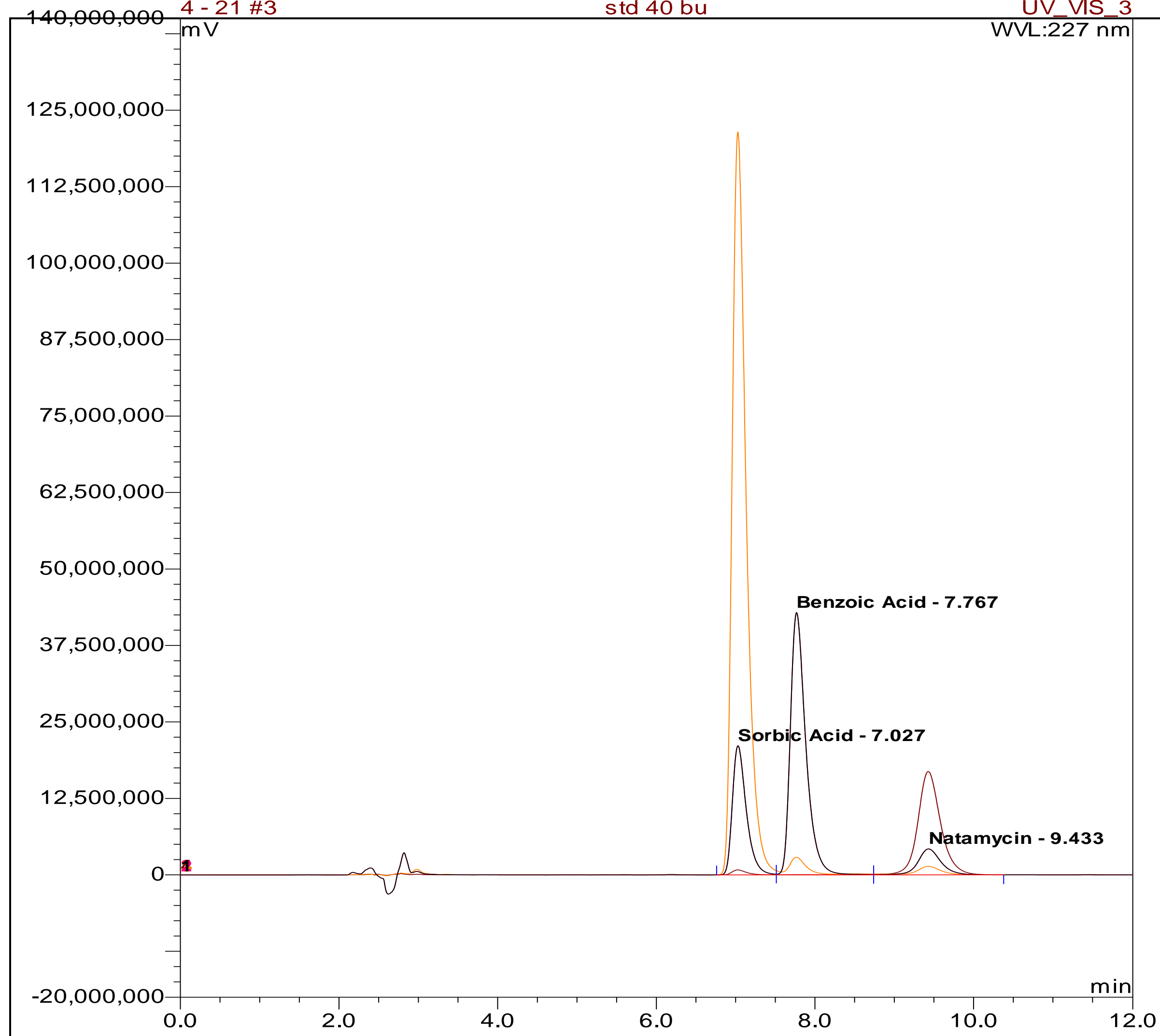

Figure 1. HPLC chromatogram of preservatives standard solutions containing 40 mg/L of sorbic acid, benzoic acid, and natamycin with a retention time 7.0 min, 7.8 min, and 9.5 min of sorbic acid, benzoic acid, and natamycin, respectively.

The bottleneck of any chromatographic system is the column, where the actual separation between analytes mixture occurs. The column selection strongly relies upon the prior knowledge of the physical and chemical properties of the analyte and matrix. The column type, length, and column particle size were optimized by studying different HPLC columns under the optimized mobile phase conditions with the intention of obtaining better chromatography in the shortest analysis time. The studied columns are:

A. Thermo Scientific™ Hypersil™ BDS 5µm C8 250 x 4.6 (Shelton, USA),

B. Thermo Scientific™ Hypersil™ BDS 3µm C8 150 x 4.6 (Shelton, USA),

C. Thermo Scientific™ Hypersil™ BDS 5µm C18 150 x 4.6 (Shelton, USA),

D. Thermo Scientific™ Hypersil™ BDS 3µm C18 150 x 4.6 (Shelton, USA).

Table 1 showed that the column bears a significant effect on the retention time and sensitivity (Area %) of preservatives. Column A (BDS 5µm C8 250 x 4.6) has excellent sensitivity for sorbic acid (99%) but suffers from poor sensitivities for benzoic acid and natamycin. Also, it has an accompanied by an increase of retention times to more than 7 min that because it is a 5µm C8 column with the longest length (250 mm). On the other hand, 3 µm BDS 150 mm × 4.6 mm, (column B) shows high sensitivity for sorbic acid (100%) and natamycin (100%) and good sensitivity for benzoic acid (92%), in addition, it has a better resolution (Rs ≈ 1.85) between sorbic and benzoic acid and optimum resolution between benzoic acid and natamycin (Rs ≈ 2.84). Besides, it has the shortest retention times (less than 5 min) that because it is a 5µm C8 column with shorter length (150 mm).

Table 1. The HPLC column packing materials investigated (Sorbic Acid, Benzoic Acid, and Natamycin at 260, 227 and 303 nm at mobile phases (65:25:10 (%, v/v)) flow rate mL/ min and column temperature 20°C.

	Column	Preservative	Retention Time (min)	Area %	Resolution
A	**Thermo Scientific™ Hypersil™ BDS 5µm C8 250 x 4.6**	**Sorbic Acid**	**5.280**	**99%**	**1.80**
		Benzoic Acid	**5.580**	**87%**	**4.42**
		Natamycin	**6.560**	**91%**	**-**
B	**Thermo Scientific™ Hypersil™ BDS 3µm C8 150 x 4.6**	**Sorbic Acid**	**3.460**	**100%**	**1.85**
		Benzoic Acid	**3.727**	**92%**	**2.84**
		Natamycin	**4.260**	**100%**	**-**
C	**Thermo Scientific™ Hypersil™ BDS 5µm C18 150 x 4.6**	**Sorbic Acid**	**7.027**	**94%**	**2.27**
		Benzoic Acid	**7.767**	**100%**	**3.98**
		Natamycin	**9.433**	**84%**	**-**
D	**Thermo Scientific™ Hypersil™ BDS 3µm C18 150 x 4.6**	**Sorbic Acid**	**3.027**	**95%**	**2.04**
		Benzoic Acid	**3.280**	**92%**	**10.34**
		Natamycin	**5.067**	**86%**	**-**

In the case of C18 columns (column C and D), column C (BDS 5µm C18 150 x 4.6) was accompanied with an increase of retention times to more than 9 min besides it suffers from a significant reduction in sensitivities for natamycin (84%). Column D, which is a 3µm C18 column with 150 mm length, shows good sensitivity for sorbic acid (95%) and sorbic acid (92%) and poor sensitivity for natamycin (86%), in addition it has an optimum resolution (Rs ≈ 2.04) between sorbic and benzoic acid with second shortest retention time to less than 6 min. Therefore, column B has been selected as it has the highest sensitivity as well shortest retention time. The optimized conditions that were achieved resulting in an effective separation of the targeted preservatives in a run time of 5 min. The average retention time was 3.4 min for sorbic acid, 3.8 min for benzoic acid, and 4.3 min for natamycin.

To assess whether the preservative mix standards could be distinguished and well separated from the interfering substances in the sample matrix, an adulterated yogurt sample was pretreated using the modified sample preparation method and separated using a BDS 3µm C8 150 x 4.6. The chromatograms that indicate the selectivity of the procedure is shown in Fig. 2. Of the chromatograms of the adulterated yogurt sample, it is evident that the peaks of the preservative standard are well separated from the peaks of the interfering substances in the sample matrix with reasonable retention times were gained.

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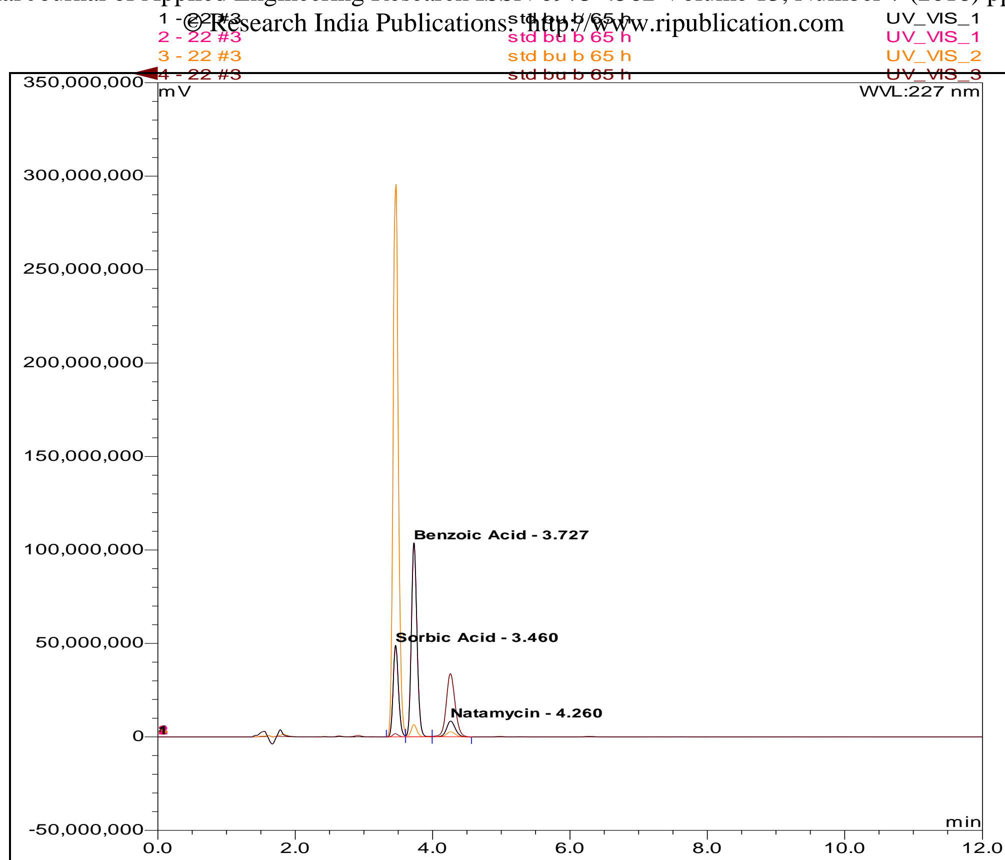

Figure 2. Representative HPLC chromatogram of a yogurt sample spiked with 5 mg/L of sorbic acid, benzoic acid, and natamycin.

Optimization extraction conditions

To perform the simultaneous detection of sorbic acid, benzoic acid, and natamycin in domestic yogurt, the extraction technique must be able to do effectively extracting every preservative from complex samples with various physicochemical properties. In general, extraction methodology is the most important part, since it must have the capacity to perform a good recovery of numerous compounds that are adulterated with preservatives [29]. Products such as yogurt with high protein and fat contents need more treatment.

Most methods currently applied in extracting preservatives use complicated, time-consuming and labour-intensive pre-treatment procedures such as employing a further precipitating step, steam distillation multiple steps or solid-phase extractions clean up step. Alternatively, these steps have been eliminated in the study due to consistent obtained chromatographic responses, thus reducing the sample treatment time and cost per analysis. Furthermore, the total extraction time for one sample is about 10 min, so this approach could be used in screening methods to achieve a fast and reliable way for the detection of the target preservatives in food.

In this procedure depicted here, the extraction solvent from the previous published benzoic acid and sorbic acid method which was mixture contains 67 mM of acetate buffer solution and methanol (67:33 (%, v/v)) was compared the mobile phase (composed of trifluoroacetic acid 0.1 % in water, trifluoroacetic acid 0.1% in acetonitrile, and trifluoroacetic acid 0.1 % in tetrahydrofuran (60:20:20 (%, v/v)).

Table 2 shows the applied mobile phase as a proposed extraction solvent in which significant enrichment were observed, around 1000 times more sensitivities (peak area) for sorbic acid. On the other hand, it did suffer from a reduction in sensitivities (peak area) of 54% for benzoic acid and shows no effect on sensitivity (peak area) for natamycin. Our goal is to have a comparable sensitivity (peak area) for all targeted analysis and to minimize sample preparation time, the mobile phase was applied as an extraction solvent in addition to been used as a mobile phase in analysis.

Table 2. 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

Method validation

The method was validated internally regarding linearity, accuracy, intra-day precision, inter-day precision, limit of detection (LOD), and limit of quantification (LOQ). The linearity was tested using the mix of preservative standards in

a concentration range from 3.12 to 50.0 mg/L for sorbic, and benzoic acids and from 2.50 to 40.0 mg/L for natamycin.

Table 3 shows good linear relationships between the concentration of the analyte and the peak response with correlation coefficients greater than 0.997 for all analytes. Calibrations with standard solutions were used for quantitation because moderate signal suppression was noticeable for both analytes. Furthermore, the ANOVA test did not give any significant difference at p = 0.05.

Table 3. Linearity range, Equation, r^2 value, LOD and LOQ of sorbic acid, benzoic acid, and natamycin.

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 accuracy was calculated by the determination of the recoveries of the preservatives from wet yogurt samples and diluted - yogurt samples at 5, 10, and 20 mg/L of sorbic acid, benzoic acid, and natamycin standards; the spiked samples were analyzed in triplicates (Table 4) and calculated according to the following formula [30]:

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

The obtained recovery percentages ranged from 81% to 111%, with a relative standard deviation (RSD) of less than 9%. The recoveries for benzoic acid were slightly more significant than the sorbic acid.

The sensitivity was determined by estimating the limit of detection (LOD) and limit of quantification (LOQ). LODs and LOQs were calculated experimentally as the lowest concentration giving a response of three- and ten-times, respectively, the base-line noise given by the software, obtained from mycotoxin-free samples. The LOD of sorbic acid, benzoic acid, and natamycin were 0.66, 0.51, and 0.01 mg/L, respectively and the LOQ were 1.3, 1.0, and 0.02 mg/L for sorbic acid, benzoic acid, and natamycin, respectively (see the details in Table 3).

Intra-day precision was calculated by assaying five replicates of the same sample at a spiked level of 20 mg/L of sorbic acid, benzoic acid, and natamycin on the same day. For the inter-day precision, five replicates of the same sample at a spiked level of 20 mg/L of sorbic acid, benzoic acid, and natamycin were analyzed on three consecutive days. The intra-day precision and inter-day precision were calculated and tabulated in Table 5. The intra-day precision (n = 5) values were between 8.4 and 8.5%, while the inter-day variation (n=15) values were between 10.4 and 11.0%. These values determined are lower than the acceptable maximum of 11%, confirming the good reproducibility and repeatability of this method.

Table 4. Mean of recoveries and RSDs (n=5) of sorbic acid, benzoic acid and natamycin spiked into clean yogurt and diluted - yogurt samples at three spiking levels using HPLC method

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**

Considering the data obtained from the method validation, the current HPLC–UV analysis measured with the aid of response surface methodology, experimental design, and sample preparation procedures is considered as a selective, precise, and robust method to determine sorbic acid, benzoic acid, and natamycin preservatives in yogurt samples.

Table 5. The intra-day precision and inter-day precision of sorbic acid, benzoic acid, and natamycin expressed as RSD% values

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

CONCLUSION

A simple, rapid, inexpensive, and effective sample preparation method has been developed for the determination of sorbic acid, benzoic acid, and natamycin in yogurt product. The sensitivity of the HPLC–UV instrument could be significantly enhanced by optimizing the mobile phase composition and the type, length, and particle size of HPLC column. The developed sample preparation procedure is based on a single extraction step without employing pre-treatment processes. Therefore, it can be recommended as an alternative to the time-consuming precipitating step, steam distillation multiple steps or solid-phase extractions clean up step for preservatives determination in food. Separation of the sorbic acid, benzoic acid, and natamycin with higher selectivity and sensitivity, and within shortened retention time was performed by a C8 150 mm × 4.6 mm x 3 µm column at 20ºC. Excellent linearity, high recoveries, acceptable repeatability, and reproducibility with lower LOQ values were succeeded indicating the suitability of the developed method for the determination of preservatives in yogurt product.

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