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Determination of Propionates and Propionic Acid in Bread Samples Using High Performance Liquid Chromatography

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----------**ABSTRACT**----------
In the present study, a HPLC method for determination of the preservative propionates in 7 groups of industrial bread samples is described. The separation the propionates were performed on the C18- column and Na2SO4 (8.0 mM) + H2SO4 (1.0 mM): acetonitrile (90:10, v/v %) as mobile phase. The detector wavelength was set at 210 nm. Separation of the propionates was achieved in less than 8 min. The samples first were milled and then extracted with 0.1 mol L-1 NaOH solution under ultrasonic irradiation. After centrifuge, supernatant clear solution was filtered using 0.45 µm Nylon syringe filter and 25 µL of solution was injected to HPLC loop. Analytical characteristics of the method such as limit of detection (LOD= 5 mg L^{-1}), recovery percentage (>91%) and reproducibility (RSD=3.5 %) were evaluated. A plot of peak area (y) versus concentration of propionate (x, mg Kg^{-1}) was linear over 10-1000 mg Kg^{-1}. The calibration graph can be described by the equation y = 301.64x + 9963 (r^2= 0.991). The levels of propionates in bread samples ranged from 3683-4752 mg Kg-1. The results stated that High performance liquid chromatography is a simple and rapid method for the determination of propionates in bakery products.
Keywords *- Bread samples, High performance liquid chromatography, Propionic acid, propionates*.

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I. INTRODUCTION

Food additives are approved for a number of different uses. Some improve the nutritional value of certain foods while others are used to make foods more appealing by improving their taste, texture, consistency or colour. Preservatives are food additives which are used to help keep food wholesome and appealing while en route to markets which may be thousands of kilometres away from where the food is grown or produced. Preservatives also help extend the storage life of foods in the home. Propionates are approved preservatives in strictly limited amounts in a range of baked foods. Foods permitted to contain propionates include bread, biscuits, cakes, pastries and other flour products [1]. Sodium propionate (SP), calcium propionate (CP) and potassium propionate (PP) are sodium, calcium and potassium salts of propionic acid. Calcium propionate is an approved preservative in bread and helps to keep the bread fresh [2]. Potassium propionate and sodium propionate are also approved preservatives [3,4]. By inhibiting the growth of mould and other microorganisms, propionates allow consumers the convenience of keeping soft, fresh bread in the home without having to purchase it every day. Although both calcium propionate and sodium propionate are equally effective anti-microbial agents, calcium propionate is the form commonly used throughout the world as a preservative in bread production [5,6]. Currently, propionic acid - propionates (E 280- 283) are authorised food additives in the EU with maximal permitted levels (MPLs) ranging from 1000 to 3000 mg/kg in foods but Turkoglu research on evaluation of genotoxic effects of sodium propionate, calcium propionate and potassium propionate on the root meristem cells of Allium cepa showed that SP, CP and PP, which are used commonly in the food industry have clear chromotoxic effects [7]. For this reason it is necessary to be careful when using these chemicals as food additives. Thus, it is necessary to develop accurate and reliable analytical methods for the confirmative determination of propionates in foodstuffs of various matrices to ensure food safety and consumer health.

In order to evaluate food safety several analytical methods have been reported for quantitative determination of propionic acid in different matrices, including gas chromatography [8-10], capillary electrophoresis [11] and high performance liquid chromatography with UV detection (HPLC–UV) [12].

The objective of the present work was the development and optimization of a sensitive, simple and fast method for determining propionates as propionic acid in some bread samples based on ultrasound-assisted extraction followed and HPLC-UV analysis for the first time. Several experimental parameters that influence the propionic

acid extraction performance were investigated and optimized. Finally, the proposed method was applied to determination of the propionic acid in some industrial bread samples in order to evaluation of propionic acid levels of them in compliance with established maximum permitted level by Codex Alimentarius.

II. EXPERIMENTAL MATERIALS AND METHODS

2.1. Chemicals and reagents

The food preservatives propionic acid, sodium propionate, calcium propionate and potassium propionate were obtained from Sigma–Aldrich (Steinheim, Germany). HPLC-grade acetonitrile was obtained from Merck Company (Darmstadt, Germany). Analytical grade sulfuric acid and sodium sulfate were also purchased from the same company. Water was purified using a Milli-Q ultrapure water purification system (Millipore, Bedford, MA, USA). All the stock solution (10000 mg L^{-1}) were prepared in water. Subsequently, working solutions (200 mg L^{-1}) were prepared from stock solutions by diluting with water. Further, Carrez I solution was prepared by dissolving 15.0 g potassium hexacianoferratein 100 mL deionized water, and for preparing 100 mL Carrez II solution, 22.0 g zinc acetate was mixed by 3.0 mL acetic acid in water.

2.2. Apparatus

The chromatographic analysis was carried out in a WellChrom Knauer high-performance liquid chromatography (Berlin, Germany) consisting of a degasser, quaternary pump (model K1100),manual sample injector with 20 µL loop size, and UV detector (model K2600) controlled by EZChrom software. The column temperature was adjusted at room temperature. Separations were carried out on a Supelcosil LC-18column (250mm × 4.6 mm, with 5 mm particle size) from Supelco Company (Bellefonte, PA, USA).. Sample data collection was optimized to 10 min per sample at 210 nm Mobile phase used was a combination of Na2SO4 (8.0 mM) + H2SO4 (1.0 mM) (phase A) and acetonitrile (phase B); 10:90 (v/v). The mobile phase was filtered through a 0.45 µm pore size filter (Merck Millipore, Billerica, Massachusetts, USA) and degassed by vacuum prior to use. Moreover, the mobile phase flow rate was set to 1.0 mL min-1. Under an optimum condition, the retention time of the propionic acid was 6.5 min.

2.3. Sample preparation

In order to extract propionic acid, the bread samples were prepared as follows: first, bread sample were dired in oven at 40 ^{0}C for 6h. Then, whole of the bread was milled and homogenate then 1.0 g of the sample weighed and then transferred to into a 50 mL beaker. Then, 10 mL of extraction solvent (H_2O:acetonitril; 50:50; v/v%) was added. Then, the suspension is placed in an ultrasonic bath for 10 min. Then, it was centrifuged for 10 min at 4000 rpm. Afterwards, the appropriate amount of the supernatant was collected and filtered through a 0.45 µm nylon membrane syringe filter, and injected into the HPLLC system.

III. RESULTS AND DISCUSSION

3.1. Optimization of sample pretreatment

Effect of extraction solvent composition and ultrasonic irradiation

Extraction of the propionates from the samples should be undertaken prior to chromatographic analysis. Three extract solvents, including water: acetonitrile 90:10, 75:25 and 50:50 (V/V) were observed to be examined in preliminary tests. Results showed similar extraction efficiency in terms of recovery and reproducibility but higher percentage of acetonitrile leads to clear chromatogram with lower interference which is favorable. In this study, the solution of 50:50 (V/V) was used in the experiments.

Then, in order to achieve the highest release efficiency of propionates from the bread matrix, the suspension is placed in an ultrasonic bath. Ultrasound assisted solvent extraction (UASE) is an inexpensive, simple, and efficient extraction technique for solid and semi-solid samples. The enhancement in extraction obtained by using ultrasound is mainly attributed to the effect of acoustic cavitations. It also exerts a mechanical effect on the texture of the sample, which allows greater penetration of solvent into the matrix and increases the contact surface area between the solid and liquid phases. As a result, the solute quickly diffuses from the solid phase to the solvent [13]. The results showed that, by applying ultrasonication for 10 min, recovery for the propionic acid could be increased up to 30% and reproducibility could be also improved.

3.2. Analytical performance

3.2.1. Linear range

The linearity for each compound was checked by analyzing mixed standard solutions of 8 different concentrations (10, 25, 50, 100, 200, 400, 600, and 1000 mg L^{-1}). The method was linear in the range of 10-1000 mg L^{-1} with regression coefficient (r^2) higher than 0.99.

Sample ID	Propionic acid level (mg Kg^{-1})[a]

3.2.2. Precision

The method exhibited excellent precision. Fortification/recovery experiments resulted in low intra day relative standard deviations (R.S.D.s) for propionic acid (n =6, R.S.D.s < 10%). A comparison of fortification/recovery experiments conducted on six different days (n = 6) also displayed low inter-day R.S.D.s (<9%), confirming the excellent reproducibility of the method.

3.2.3. Detection and quantitation limits

Detection limits were calculated by extracting diluted solutions of the analyte (0.5, 1, 2.5 and 5.0 mg L^{-1}). The criteria were selected according to IUPAC and ACS definition, as follows:

Detection limit (DL): A_s-A_b=3S_b

Quantitation limit (QL): A_s-A_b=10S_b

were A_s is the average of sample signal (area); A_b is the average of blank signal (area) and S is the standard deviation of blank signal (area). The obtained DL was 5.0 mg L^{-1}.

3.2.4. Accuracy

Due to the lack of Certified Reference Materials, accuracy evaluation, in terms of percentage of recovery, was carried out on blank samples spiked with a known amount of analyte (level of 500 mg L^{-1}). Data for these experiments are shown in Table 2. Good results were obtained, with average recoveries ranging from 91.0 to 103.4%. It is interesting to note that, samples did not require matrix matched calibration curves to compensate for difficulties in measuring peak area for propionic acid at low concentration, making more rapid analysis possible.

3.3. Application

The validated method was applied to determine content of propionates in different bread samples collected locally and the results are summarized in Table 1. Also, histogram of the obtained results is shown in Fig.1. RSD values for three replicate measurements for all of the tested samples were less than 8.0%. In the analysis of the samples, peak identification was based on the comparison between the retention times of standard compounds and was confirmed by spiking standards to the samples. Quantification was based on the external standard method using calibration curves fitted by linear regression analysis. Under the stated experimental conditions, baseline separation of the propionates was achieved in less than 8 min. A typical HPLC-UV chromatogram of a bread sample is shown in Fig. 2 before (Fig. 2a) and after spike 500 mg Kg^{-1} (Fig. 2b). Statistical results of the propionic acid amount in studied bread samples (Table 4) showed that the propionic acid concentration of the 30 bread samples range between 2852-6993 mg Kg^{-1}. Also, the average and median of the results were 3952 and 3686 mg Kg^{-1}, respectively.

Levels of propionic acid and propionates are higher than maximum allowable level of the Iran and Codex Alimentarius legal limits of 1500 mg Kg^{-1} (Table 4). According to obtained results from one sample T-test, at 95% Confidence Interval, there is significant diffence between propionic acid levels of the tested samples and the established Codex Alimentarius legal limit [1].

Bread 1	3824
Bread 2	2962
Bread 3	3419
Bread 4	3172
Bread 5	3124
Bread 6	3535
Bread 7	3414
Bread 8	5156
Bread 9	4550
Bread 10	4340
Bread 11	3378
Bread 12	6993
Bread 13	4975
Bread 14	4125
Bread 15	3383
Bread 16	3879
Bread 17	4399
Bread 18	3685
Bread 19	4445
Bread 20	3154
Bread 21	4332
Bread 22	3300
Bread 23	4406
Bread 24	3641
Bread 25	3005
Bread 26	4785
Bread 27	2852
Bread 28	3688
Bread 29	5345
Bread 30	3293

Table 1. Levels of Propionic Acid Found in Different Bread Samples (n = 3) Using the Proposed HPLC Method

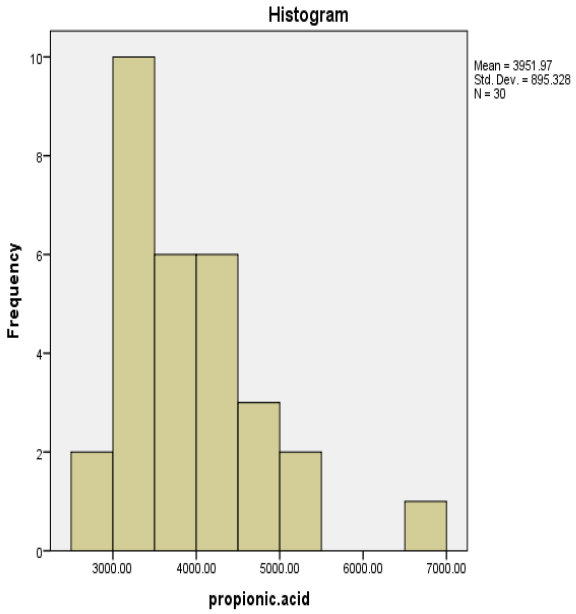


Fig. 1. Histogram of Variation of the Propionic Acid Amount in Studied Bread Samples

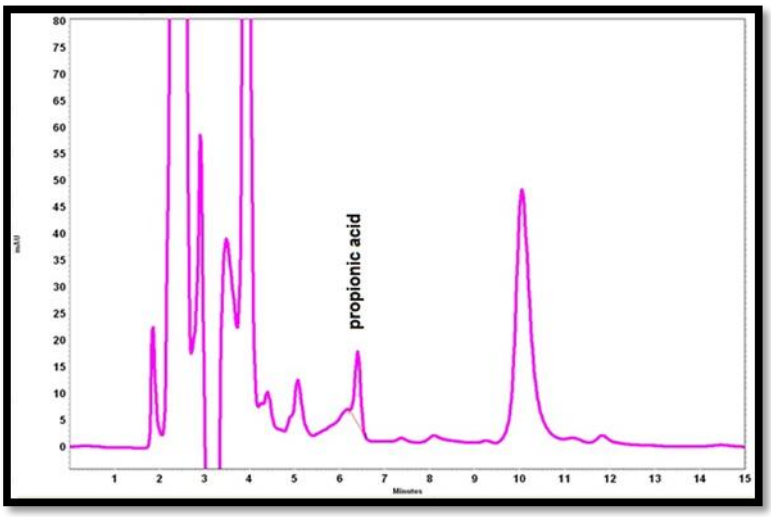
Table 2. Statistical Results of the Propionic Acid Amount in Studied Bread Samples

Descriptives				
			Statistic	Std. Error
propionic.acid	Mean		3951.9667	163.46385
	95% Confidence Interval for Mean	Lower Bound	3617.6456	
		Upper Bound	4286.2878	
	5% Trimmed Mean		3872.6111	
	Median		3686.5000	
	Variance		801612.930	
	Std. Deviation		895.32839	
	Minimum		2852.00	
	Maximum		6993.00	
	Range		4141.00	
	Interquartile Range		1117.50	
	Skewness		1.481	0.427
	Kurtosis		3.168	0.833

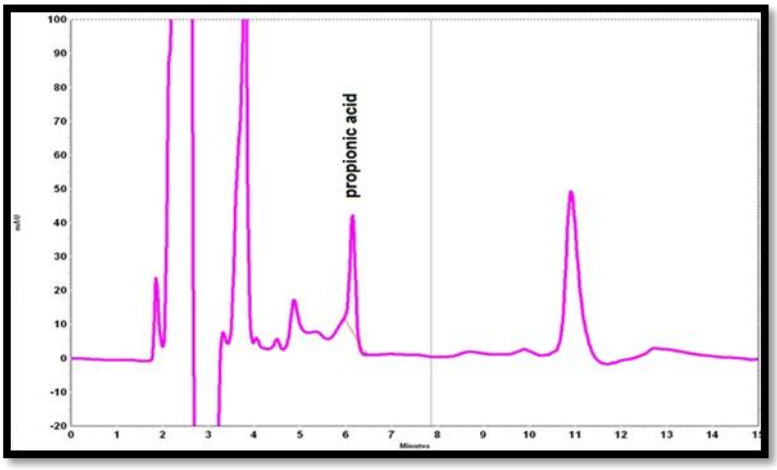
Table 3. Results of One Sample t-test (R* =1500 mg Kg^{-1})

	95% Confidence Interval of the Difference		Mean Difference	Sig (2-tailed)	df	t
upper	lower					
2786	2117		2452	0.00	29	15

* Maximum allowable limit of propionic acid level according to CODEX Alimentarius



(A)



(B)

Fig. 2. HPLC-UV chromatogram (λ =210 nm) of bread 4 for (a) nonspiked and (b) spike of 500 mg L^{-1} of propionic acid.

IV. CONCLUSION

Due to the lack of International Standard Method for the determination of the propionates in foodstuff specially bread samples, in this research we propose an analytical method for the determination of the propionates in different industrial bread samples. Proposed method is simple, rapid, reliable and allows good recoveries of the analytes in the tested samples. The performance of the whole UASE-HPLC-UV method showed its suitability for an accurate quantification of propionates at levels as low as 10 mg L^{-1}, so it can be used for routine control of bread samples based products to evaluate the current maximum limit of 1500 mg Kg^{-1} set by Codex Alimentarius [1]. Except one sample, samples analyzed showed values higher than the maximum limit (in most of the samples 2-3 times higher than maximum limit).

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