

**Research Article****Determination of stearoyl lactylate in starchy products using liquid chromatography method after hydrazine derivation**

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**Abstract**

Sodium stearoyl lactylate (SSL) is an emulsifying agent that significantly enhances dough volume, surface area, and structural integrity while also increasing air retention even with a small amount of yeast added. Its remarkable properties extend to improving preservation quality, facilitating finer slicing, and inhibiting microbial proliferation, thereby reducing shortening. Owing to these benefits, SSL finds extensive application in the production of starch-containing industrial products. In this study, SSL was quantified following the hydrazine derivatization stage using the HPLC-PDA method. The lactate derivative was analyzed on a C18 column with a gradient program comprising 0.1% formic acid and acetonitrile. The method was validated and met the AOAC requirements for good linearity ( $R^2 = 1$ ). The limits of detection (LOD) and limits of quantification (LOQ) were 2.00 mg/kg and 6.66 mg/kg SSL for bread samples; 1.45 mg/kg and 4.82 mg/kg for biscuit samples. Precision was performed on spiked bread and biscuit matrices with relative standard deviations of repeatability  $RSD_r = 1.91\%$  and  $3.07\%$ , respectively; the relative standard deviation of reproducibility ( $RSD_R$ ) was  $4.22\%$  and  $3.47\%$ . Recovery values ranged between  $95.1\%$  and  $105\%$  for bread and  $95.4\%$  to  $104.7\%$  for biscuits. The validated method was subsequently applied to analyze SSL content in 20 different starchy products purchased in the Hanoi market.

**Keywords:** Stearoyl lactylate, SSL, nitrophenyl hydrazine, starchy products, HPLC-PDA.

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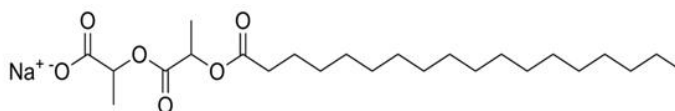
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## 1. INTRODUCTION

Sodium stearyl lactylate is a mixture of the sodium salt of stearyl lactic acid, depicted in Figure 1, along with its polymers, and small amounts of sodium salts of other related acids, produced by the reaction of stearic acid and lactic acid [1].

Sodium stearyl lactylate is soluble in oils and fats, processing the ability to attract water molecules, so it is useful in creating oil-in-water emulsions and preserving the moisture content of food products. Furthermore, the interaction of SSL and starch and its capacity to form complexes with proteins contribute to its widespread application as dough conditioner/emulsifier in leavened baked goods helping increase leavening and soften the crumb, increasing dough volume and dough strength and symmetry of bread, creating a crispy crust. Besides this, SSL is also used as a foaming agent in both dairy-based whipped desserts and dairy-free coffee creamers, where it acts as a surfactant and chelating agent, as well as in whitening powders as fat substitutes [2, 3].

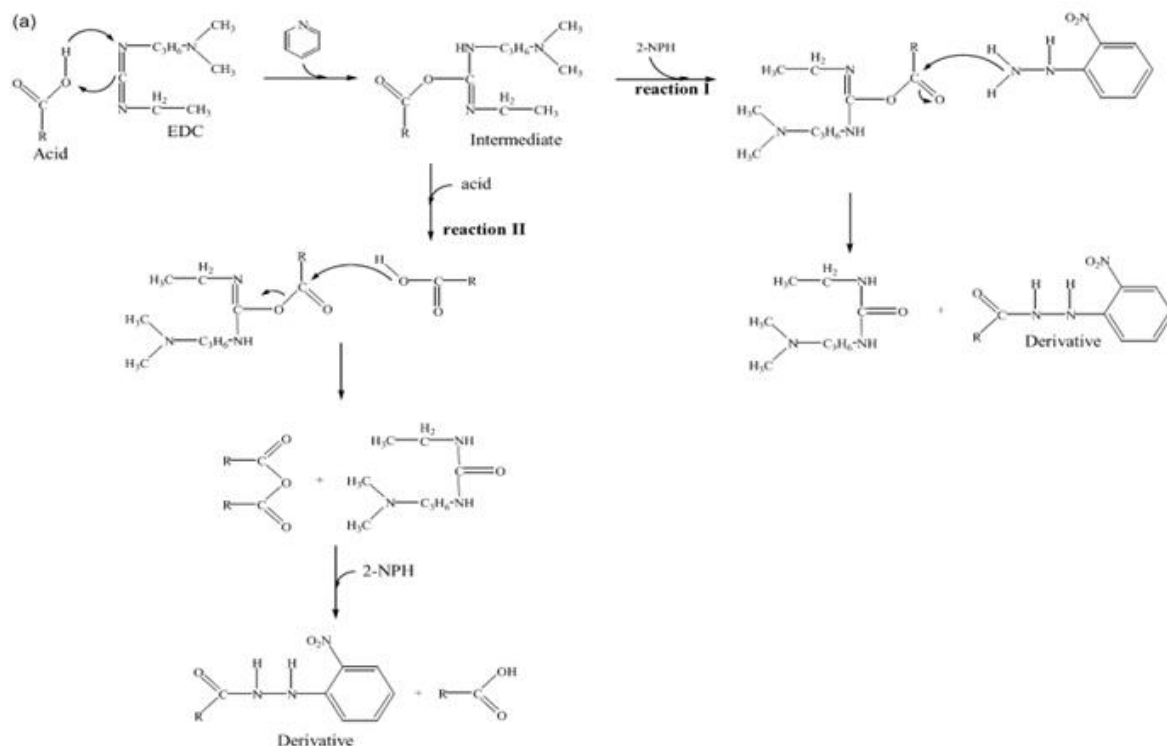


**Figure 1.** Structural formula of SSL

Studies evaluating the physiological impact of SSL showed some side effects of SSL such as mild respiratory irritation upon inhalation of its pure form. Toxicity assessments gave a median lethal dose (LD50) exceeding 25 g/kg body weight with a no-observed-adverse-effect level (NOAEL) set at 5% of the diet for SSL or Calcium stearyl lactylate (CSL). According to documents from EFSA and the Scientific Committee on Food (SCF), the acceptable daily intake (ADI) is 20 mg/kg body weight/day for SSL and CSL. Estimated exposure to sodium stearyl-2-lactylate occurs primarily through consumption of bread and bakery products. In the United States, the maximum level of use of SSL in some foods is allowed from 0.2 - 0.5%, while in Europe it is allowed from 2000 - 8000 mg/kg for specific products [4, 5].

In assessing the proper utilization of SSL and ensuring compliance with regulatory standards for commercial additive formulations, various analytical methods have been developed such as GC-FID [6-9], TLC thin layer chromatography [10, 11], liquid chromatography-mass spectrometry LC-MS [11-13], or liquid chromatography with PDA detector [8, 14, 15]. However, the TLC method is not highly appreciated for its limited sensitivity or accuracy. While GC-FID and LC-MS methods are expensive. Moreover, GC-FID base on retention time for identifying analytes, which easily causes peak confusion. On the other hand, LC-MS are highly affected by sample matrices so the internal standard is required. The preferred method for quantitation of SSL from lactic acid ratio by HPLC due to its simplicity and widespread availability. Lactic acid shows not enough for UV-Vis detection and is usually quantified at a low wavelength of 210 nm, resulting in poor specificity and selectivity, especially in food samples with low lactic acid content [15]. However, derivatization of lactic acid with 2-nitrophenyl hydrazine generates a derivative with a characteristic absorption wavelength at 400 nm, overcoming the above disadvantages

and ensuring specificity, sensitivity and accuracy. Thus, the HPLC method with hydrazine derivative step proves suitable for the analysis of SSL content in food samples [8, 14, 15]. The reaction mechanism of lactic acid- nitrophenyl hydrazine derivatization is described in Figure 2 [15]. There are 2 theoretical reaction pathways for derivatizing lactic acid but reaction I was the main.



**Figure 2.** Mechanism of lactic acid- nitrophenyl hydrazine derivative

Hence, the objective of this study is to formulate a HPLC method with 2-nitrophenyl hydrazine derivation step to determine lactic acid originating from sodium stearoyl lactylate (SSL) in foods derived from starch. The developed method has the advantage over the published method [8, 14, 15] that it employs C18 columns that are highly available in laboratories while previous studies used less common such as C18 XDB and C8 columns. Additionally, replacing the nitrogen flow drying step with vacuum evaporation enhances efficiency and cost-effectiveness.

## 2. MATERIALS AND METHODS

### 2.1. Equipment

The main equipment is HPLC connected to the PDA detector used in the research provided by Shimadzu, model: 20A. Other apparatus includes Analytical balance from Mettler Toledo (Switzerland); Technical balance from Sartorius (Germany); Vacuum evaporation system equipped with water bath; Thermal bath from Memmert (Germany) capable of temperature adjustment; SPE solid phase extractor and pressure pump and sample homogenizer.

## 2.2. Chemicals and materials

Standard substances used included sodium stearoyl lactylate (SSL) purity  $\geq 97\%$  and lithium L-lactate ( $\geq 98\%$  purity) from Sigma Aldrich. Chemicals and solvents consisted of 2-nitrophenylhydrazine (2-NPH) purity  $\geq 97\%$  with 30% water (China); 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) from Thermo Fisher Scientific; 36.5% HCl solution (Merck); KOH (Merck); Pyridine (China). Other solvents were n-hexane, chloroform, ethyl acetate, ethanol, methanol (Merck) with chromatographic purity; Deionized water; InertSep C18 column 500 mg/6mL (GL sciences, Tokyo, Japan) and 0.22  $\mu\text{m}$  filter membrane.

## 2.3. Experiments

### 2.3.1. Preparation of standard solution

Lactic acid standard solution is prepared from the corresponding lithium L-lactate standard in deionized water. The solution is stored in the refrigerator. Working standard solutions with concentrations of 0.5 - 200 mg/L were prepared daily from a 1000 mg/L stock standard solution.

### 2.3.2. Preparation of other solvents and reagents

1% KOH solution was prepared in ethanol, 5% KOH solution was prepared in ethanol: water (1:1, v/v). Reagent 2-NPH-HCl (0.02 M) was mixed in 0.3 M HCl solution in ethanol (1:1, v/v). EDC-HCl reagent (0.25 M) was dissolved in 3% pyridine solution in ethanol (v/v).

### 2.3.3. Sample collection

20 starch-based food samples including 03 dumplings samples (M2, M3, M5), 04 cake samples (M1, M7, M9, M13); 06 bread samples (M4, M8, M11, M17, M19, M20); 02 instant fried noodles products (M10, M18); 01 fresh noodles (M14); 02 wafers samples (M15, M16) and 02 traditional products (M6, M12) were collected from supermarkets or bakery in Hanoi.

### 2.3.4. Sample preparation

The sample preparation process is shown in Figure 3.

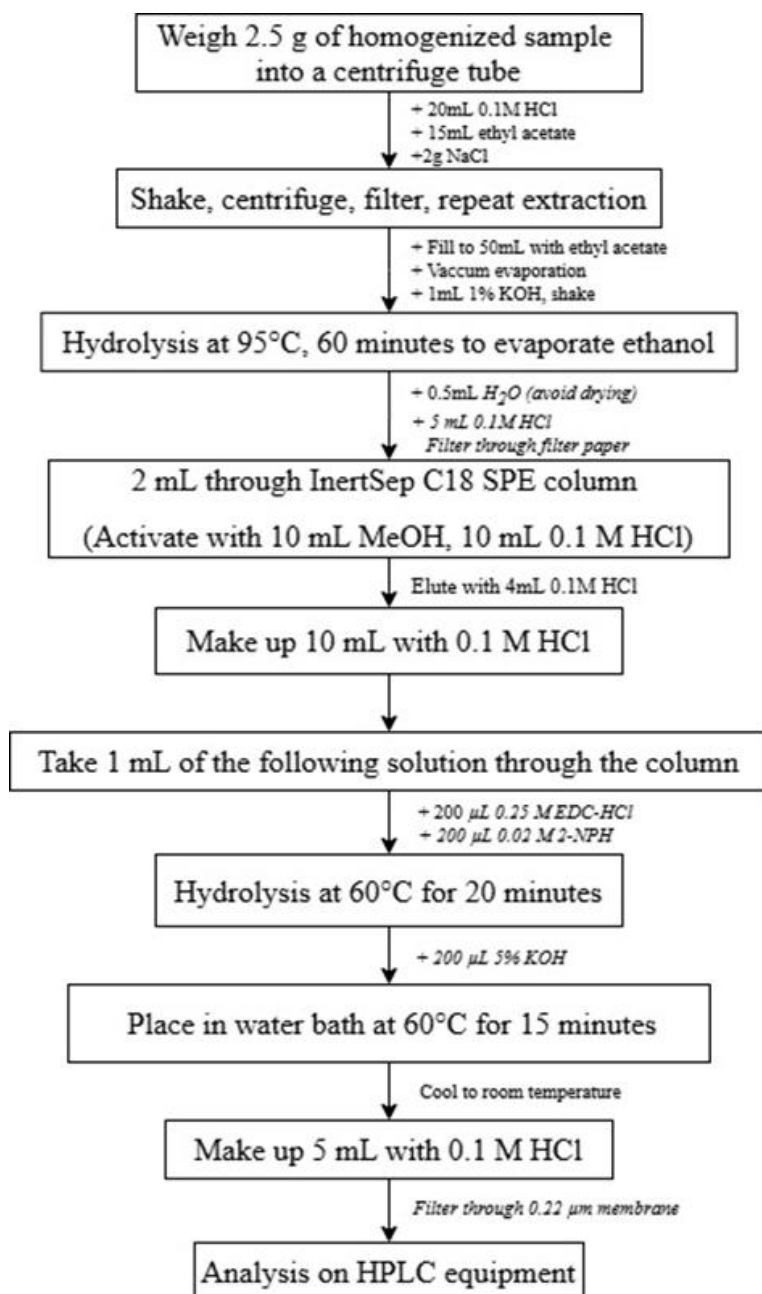
### 2.3.5. Chromatography conditions

The analytical procedure was conducted using a High-Performance Liquid Chromatography-Photodiode Array (HPLC-PDA) system, operating at a wavelength of 400 nm. The separation was achieved on a Reliant C18 column (5  $\mu\text{m}$ , 4.6 mm x 250 mm). The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B), delivered at a flow rate of 0.8 mL/min. The initial gradient condition was set at 90:10 (A:B, v/v), which was then modified to 70:30 (v/v) over a period of 5 minutes, maintained for an additional 3 minutes, and subsequently returned to the starting ratio by the 12-minute mark. The total runtime for the analysis was set at 15 minutes, with an injection volume of 20  $\mu\text{L}$ .

### 2.3.6. Method validation

The method was validated on blank bread and biscuit matrices for the specificity, linear range, sensitivity, precision, and accuracy according to AOAC International Guidelines for standard method performance requirements [16]. The specification of the method was

compared over the retention time of lactate-NPH responses in the blank bread sample, standard, and blank bread sample spiked with SSL standard. The limit of detection (LOD) and limit quantification (LOQ) were determined by analyzing 10 times SSL spiked samples at low concentrations (10 mg/kg) and calculated by multiplying 3 times of SD value for LOD and 10 times for LOQ. Sodium stearyl lactylate standard was added at 3 concentration levels 1mg to 4 mg to blank samples of bread and biscuit, repeating the analysis 3-6 times to assess the recovery. The repeatability and reproducibility were conducted by analyzing 6 times ( $n = 6$ ) and 4 times ( $n = 4$ ) of the spiked bank bread sample at 1.0 mg and the spiked blank biscuit sample at 1.5 mg SSL.



*Figure 3. Flow chart of sample preparation*

### 2.3.7. Real sample analysis

Using the developed method for analyzing 20 sample products collected in Hanoi's markets. SSL content was calculated from the content of lactic acid which is derived from sodium stearoyl lactylate and sodium palmitoyl lactylate which is described in the formula:

$$\text{SSL content} = C \times (100/20)$$

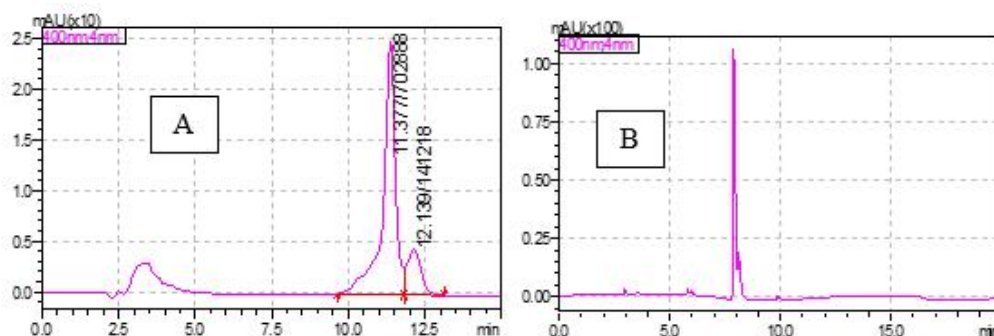
in which C is lactic acid content calculated from the calibration curve of lactate-NPH; 100/20 was the 20% approximately ratio of lactic acid derived from sodium stearoyl lactylate and sodium palmitoyl lactylate [14].

## 3. RESULTS AND DISCUSSION

### 3.1. Optimize LC conditions

#### \* *Chromatography column*

Upon reviewing articles [8, 14] and laboratory conditions, a standard lactate analysis was conducted using nitrophenyl hydrazine derivatives at a concentration of 100 mg/L (standard lactate-NPH solution). This was performed on a Symmetry C8 column (5  $\mu$ m, 4.6 mm x 250 mm) with a mobile phase of 0.1% formic acid and methanol in an isocratic mode of 75:25 (v/v), and on a Reliant C18 column (5  $\mu$ m, 4.6 mm x 250 mm) with a mobile phase of 0.1% formic acid and acetonitrile in a gradient elution program. The results in Figure 4 indicated that, with the C18 column, complete separation was not yet achieved. However, the chromatographic peaks obtained were neater and more symmetrical. Therefore, the C18 column was selected for further research.

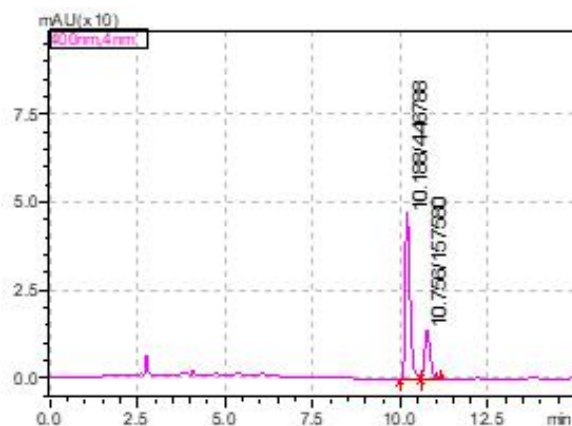


**Figure 4.** Chromatogram of lactate-NPH using C8 column (A) and C18 column (B)

#### \* *Gradient elution condition*

The less polar lactate-NPH derivatives will elute rapidly during the less polar mobile phase transition. Consequently, the ratio between the aqueous component of the mobile phase (0.1% formic acid) and the less polar organic phase (acetonitrile) was investigated to optimize the separation process for the standard derivative and the excess derivative.

With a flow rate of 0.8 mL/min and an optimized gradient program, the analytical peaks were symmetrical with the resolution factor R being 2.035 (Figure 5). Therefore, the aforementioned gradient program has been selected for SSL analysis. The optimized LC conditions for analyzing SSL are in 2.3.5.



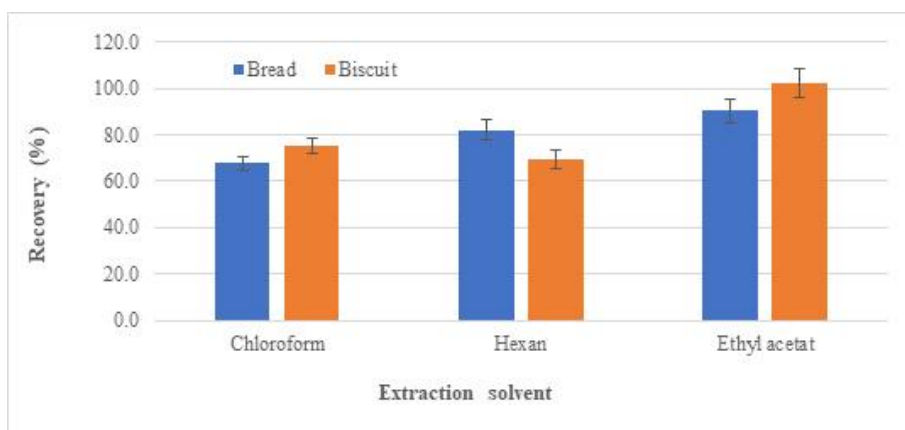
**Figure 5.** Chromatogram of lactate-NPH using optimized gradient

### 3.2. Optimize sample processing

#### \* Sample extraction solvent

The structural formula of SSL, comprising a lipophilic group that significantly affects its physical properties (long-chain stearyl base) and a hydrophilic group (ester base of lactic acid with sodium ions), allows SSL to dissolve well in less polar organic solvents or oil phases [6]. Moreover, in the analysis of SSL, the final product is calculated based on lactic acid, thus the chosen solvents must not dissolve lactic acid and lactate salts (if present) in the sample matrix. Therefore, a survey was conducted on three organic solvents commonly used in laboratories including ethyl acetate, hexane, and chloroform with two spiked bread and biscuit matrices.

The results depicted in Figure 6 indicate that for both sample matrices (bread and biscuit), ethyl acetate yielded the highest recovery efficiency, followed by hexane and chloroform. Furthermore, ethyl acetate was also assessed to be more environmentally friendly compared to the other two solvents [11]. Consequently, ethyl acetate was selected as the solvent for SSL extraction in this study. There is no free lactic acid in ethyl acetate extraction solvent in this experiment (data not shown).

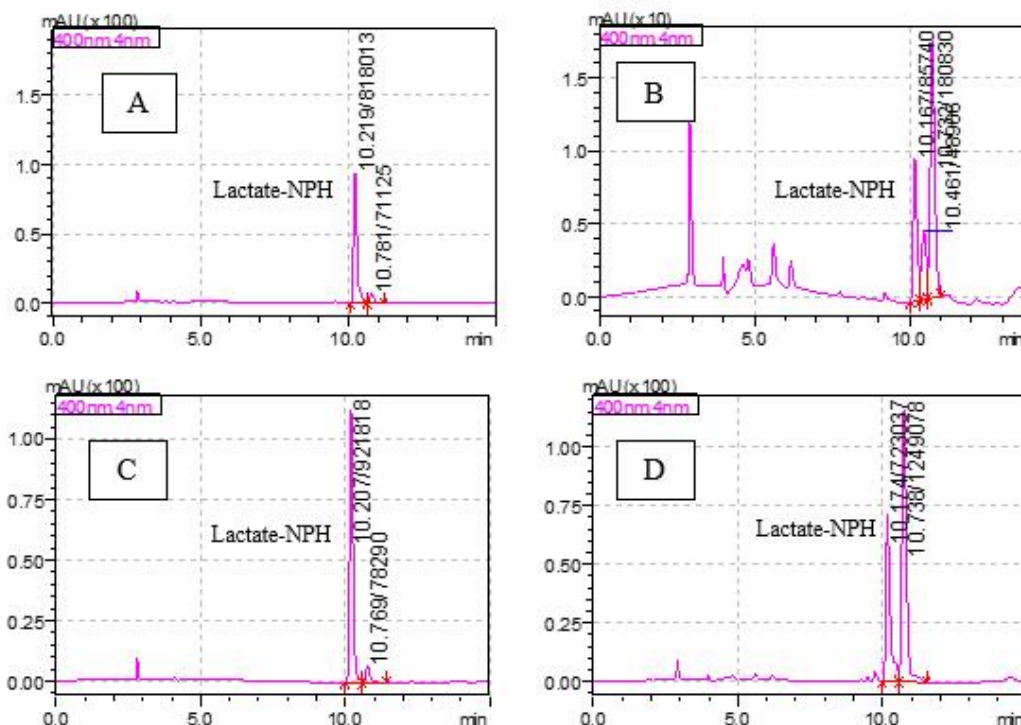


**Figure 6.** Graph of recovery according to extraction solvent

### \* *Cleaning up by SPE*

In the bread and biscuit matrices, extraction with ethyl acetate allows for the solubilization of fats and other substances into this organic phase. Therefore, a purification step using solid-phase extraction columns is necessary to remove impurities. Based on references [8, 14, 15], the InertSep C18 SPE column 500 mg/6mL (GL Sciences, Tokyo, Japan) was employed in this study.

1 mL of 150 mg/L lactate standard had passed through the InertSep C18. A recovery efficiency of 98.9% indicates the column's effectiveness for lactic acid. Comparative analysis of the bread and biscuit extracts, both pre and post-column processing and subsequent derivatization, highlighted the necessity of column purification. Samples bypassing this step displayed significant impurities, with poorly separated peaks as depicted in Figure 7. Additionally, these samples showed a diminished recovery efficiency compared to their column-processed counterparts, due to the presence of interfering substances in the turbid unprocessed solution, which adversely affected the derivatization and detector signals. Thus, the purification using the InertSep C18 column was essential for this study.

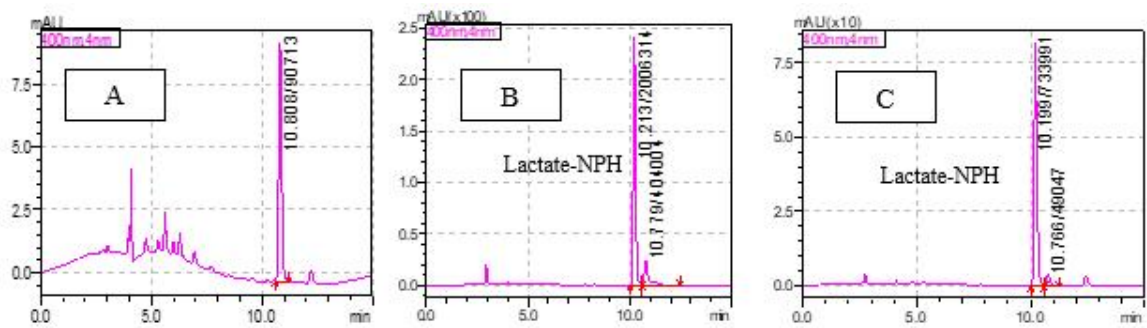


**Figure 7.** Chromatogram of purified sample and unpurified sample (A-purified bread sample; B-unpurified bread sample; C-purified biscuit sample; D-unpurified biscuit sample)

## 3.3. Method validation

### 3.3.1. Specification

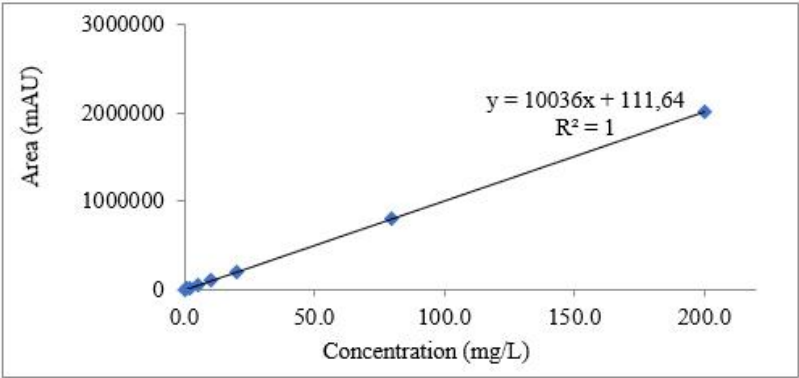
In Figure 8, there is no analyte signal in the blank sample. The retention times of the analyte in the standard and spiked samples were insignificantly different. Thus, the method has proven specificity.



**Figure 8.** Specification of derivative lactate-NPH  
(A: blank bread sample; B: standard; C: blank bread sample spiked)

3.3.2. Calibration curve

Calibration curves were constructed in the range from 0.5 to 200 mg/L of lactic acid standard solutions calculated from lithium lactate. The results of the standard curve equation and corresponding regression coefficient:  $y = 10036x + 111.64$  with coefficient  $R^2 = 1$  show a good correlation coefficient (Figure 9).



**Figure 9.** Calibration curve

3.3.3. Precision, trueness, LOD, LOQ

The results of limit of detection (LOD), limit of quantification (LOQ), precision (repeatability -  $RSD_r$ , reproducibility -  $RSD_R$ ) and trueness (recovery -  $R$ ) are shown in Table 1.

**Table 1.** Method validation data

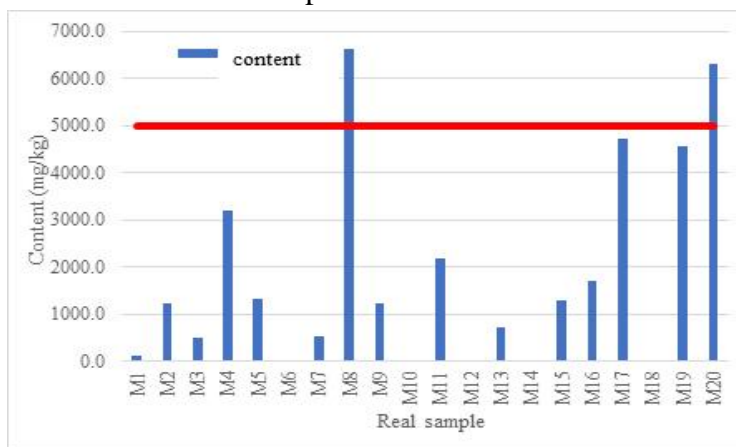
Parameter	Bread	Biscuit
LOD (mg/kg)	2.00	1.45
LOQ (mg/kg)	6.66	4.82
$RSD_r$ (%)	1.91	3.07
$RSD_R$ (%)	4.22	3.47
$R$ (%)	95.1-105.0	95.4 – 104.7

The data presented in Table 1 clearly indicate that the criteria are within the permissible AOAC thresholds, with a requisite concentration of 0.1%. The relative standard

deviation for repeatability ( $RSD_r$ ) is below 3.7%, and for reproducibility ( $RSD_R$ ) is under 6.0%. Furthermore, the recoveries are in the range of 95-105%. The validation outcomes robustly support the suitability of the HPLC-PDA technique for the analysis of SSL in starchy products [16].

### 3.4. Application on starchy product samples

Applying the validated method with optimal LC and sample preparation conditions to analyze 20 samples, which are derived from starch such as bread, wafers, dumplings, instant noodles and some Vietnam's traditional products. The results are shown in Figure 10.



**Figure 10.** Content of SSL in 20 starchy products

The results of SSL analysis in 20 products show that the samples have content in a wide range from 133 – 6610 mg/kg. Several products have quite high concentrations, ranging from 2175 to 6610 mg/kg, particularly those identified as bread products (M4, M8, M11, M17, M19, and M20). These concentrations are consistent with previous reports on SSL addition levels in some product types (approximately 0.1 - 0.5%) to achieve desired bread characteristics such as increased dough volume, symmetry and air retention [17, 18]. Dumplings, wafers, cakes displayed elevated SSL content but below the threshold reported in prior studies (0.2 - 0.5%). This is said to mean that the presence of meat, cream and cheese fillings in these products affects the overall product volume. Other products such as instant noodles (M10, M18), Vietnamese traditional products (M6, M12), and fresh noodles (M14) were all not detected the content of SSL. According to EU regulation No 1129/2011, two bread samples (M8 and M20) exceeded the Maximum Permitted Levels (MPLs) of SSL in bakery products. However, in Vietnam, it lacks of regulation for SSL content in food samples in general.

## 4. CONCLUSION

An HPLC-PDA method incorporating a 2-nitrophenyl hydrazine derivation step for quantification of SSL in starchy products was developed and validated following the AOAC guidelines for specificity, linearity, accuracy, repeatability, reproducibility, LOD, and LOQ. The validated method was applied successfully to analyze SSL content in 20 different starchy products.

## ACKNOWLEDGEMENT

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