*Available online www.jocpr.com*

**Journal of Chemical and Pharmaceutical Research, 2016, 8(4):1458-1464**

**Research Article**

**ISSN : 0975-7384**
**CODEN(USA) : JCPRC5**

# Colorimetric Method for Total Phytosterols Content Analysis in Soybean (*Glycine max*), Soymilk, and Soy Yoghurt

**Nyi Mekar Saptarini*, Wiwiek Indriyati and Ayu Shalihat**

*Faculty of Pharmacy Padjadjaran University, Jl Raya Bandung Sumedang Km 21 45363 West Java Indonesia*

---

**ABSTRACT**

*Soy yoghurt is a fermented soymilk products. The fermentation process by Lactobacillus bulgaricus and Streptococcus thermophillus change the content and composition of bioactive compounds, such as phytosterols. The aims of this study is to evaluate the colorimetric method to quantify the total phytosterols content in soybean, soymilk, and soy yoghurt. The procedure was based on the colored products of phytosterols with Liebermann-Buchard reagent. The method was evaluated for linearity, accuracy, precision, limit of detection, and limit of quantification. The procedure meets the validation criteria. Colorimetric method is a valid procedure to determine the total phytosterol content in herbal derivatives.*

**Keywords:** Liebermann-Buchard reagent, validation method, fermentation time dependent, herbal derivatives

---

## INTRODUCTION

Phytosterol is a sterol compounds in plants, such as sitosterol, stigmasterol, and kampesterol. Phytosterols have anti-atherogenic properties. Consumption of phytosterols can inhibit the cholesterol absorption, which increase the cholesterol excretion. Recommended phytosterols consumption is 2 g/day to reduce 30-40% of cholesterol absorption and 10% of LDL-cholesterol absorption [1]. The total phytosterol content of soybean is 2.5 g/kg [2].

Soy yoghurt is a fermented soymilk product with high nutritional value. During the fermentation, the organic acids are formed, which cause the specific flavor. Soy yoghurt was produced by *Lactobacillus bulgaricus* and *Streptococcus thermophillus*, which commonly used in yoghurt fermentation [3]. Long-term fermentation will increase the content of protein, fat, lactic acid and nonfat dried weight, and change the content and composition of bioactive compounds in soy yoghurt [4].

The aims of this study is to evaluate the colorimetric method to quantify the total phytosterols content quickly and easily. In this study, we determined the total phytosterols content in soybean, soymilk and soy yoghurt from the various of fermentation time. We also analyzed the effect of fermentation time to the total phytosterols content in soy yoghurt, then we characterized the soy yoghurt from the optimal fermentation.

## EXPERIMENTAL SECTION

### Materials

Soybean were obtained from Sumedang, West Java, Indonesia and identified by Plant Taxonomy Laboratory, Department of Biology, Padjadjaran University. The 95% phytosterols standard was obtained from Jiatian Biotechnology, Xi'an, China. All chemicals were analytical grade (Merck), i.e n-hexane, ethanol, ethyl acetate, acetic acid anhydrous sulfuric acid, chloroform, sodium sulfite, and sucrose.

*Streptococcus thermophillus* (ATCC 19 258) was obtained from the Microbiology Laboratory, Department of Biology, Padjadjaran University, and *Lactobacillus bulgaricus* (ATCC 11842) was obtained from the Microbiology Laboratory, Faculty of Industrial Engineering, Bandung Institute of Technology.

**Methods**

***Phytochemical screening of Soybean***

Phytochemical screening was carried out according to the color reaction [5].

***Soymilk Preparation***

Soymilk was made using the modified Illinois method [6]. Soybean were washed, then soaked for 8 hours with water, replacement of every 2 hours. Soybean were boiled on a 85-95 °C for 20 minutes, then removed and cooled. Soybean were refined with water (1: 5), then filtered.

***Soy yoghurt Preparation***

***Bacterial enrichment.*** *L. bulgaricus* and *S. thermophilus* were inoculated into the medium propagation (MRS Broth), incubated for 8 hours, and used as a pure culture.

***Starter cultures preparation.*** (i) The main culture preparation: 5% pure culture (of the skim milk volume) was inoculated in 10% sterile skim milk medium, then incubated at 37 °C for 24 hours or until the formation of curd. (ii) The starter culture preparation: 5% of the main culture was inoculated in soymilk contains 5% sterile skim milk, then incubated at 37 °C for 24 hours or until the formation of curd. This culture is the starter culture for the soy yoghurt preparation.

***Soy yoghurt preparation.*** Soy yoghurt was made using the modified Kanda method [7]. Soymilk was added with 5% sucrose and 5% skim milk. Soymilk was pasteurized at 80 °C for 30 min, then cooled to 45 °C. The 2.5% of *S. thermophillus* and 2.5% *L. bulgaricus* were inoculated, and fermented at 37 °C for 2, 4, 6, 8, 10, and 12 hours. Soy yoghurt was stored in a refrigerator at 5 °C to stop the fermentation process.

***Phytosterols Extraction***

***Soybean.*** The soybean (100 g) was extracted with 200 mL of n-hexane:ethanol (82:18) for 24 hours at 25 °C. The exract was filtered, and the residue was re-extracted twice, using 200 mL of the same solvent for 24 hours. The extract was concentrated by rotary vaporation at 40 °C, and saponified with 26.73 M KOH solution. The unsaponified phase was separated with n-hexane. Crude sterols extract was concentrated by rotary vaporator at 40 °C [8].

***Soymilk and soy yoghurt.*** The samples (10 g) were added with 10 M KOH solution in methanol (9: 1), then extracted by reflux apparatus for 30 minutes at 50 °C. The samples were cooled to 25 °C, then sterols were extracted by the liquid-liquid extraction, three times with 5 mL of distilled water and 10 mL of n-hexane. The n-hexane phase was separated, then added sodium sulfite to remove the remaining water.

***Qualitative Analysis by Thin Layer Chromatography (TLC)***

The standard and samples were spotted on a silica gel $GF_{254}$, eluted with n-hexane-ethyl acetate (4: 1), then dried the plate, and sprayed with Lieberman-Burchard reagent [9]. Color and Rf value of samples were compared to the standard.

***Quantitative Analysis of the Total Phytosterols Content***

***Solution preparation.*** (i) *Liebermann-Buchard reagent (LB):* The acetic acid anhydride is cooled for 30 min, then added concentrated sulfuric acid in the ratio 10: 1. The reagent should be fresh. (ii) *Standard solution:* The phytosterols standard (50 mg) was dissolved with chloroform in a 100 mL volumetric flask. (iii) *Sample solution:* Extract, soymilk, and soy yoghurt (5 mL), each sample was dissolved in 20 mL of chloroform using an ultrasonic bath until dissolved completely.

***Optimization of analysis conditions.*** (i) *Wavelength selection*: Standard solution (1 mL) was added to 4 mL of LB, and chloroform in 10 mL of volumetric flask. The mixture was incubated for 5 min, then measured the absorbance at 400-900 nm with spectrophotometer. (ii) *Determination of the time reaction*: Standard solution (1 mL) was added to 4 mL of LB, and chloroform in 10 mL volumetric flask. The absorbance was measured at the maximum wavelength every 5 min for 60 min. (iii) *Optimization of LB volume:* Each of five standard solution (1 mL) was added with various LB (1, 2, 3, 4, and 5 mL), and chloroform in a 10 mL volumetric flask. The absorbance was measured at a maximum wavelength after optimum incubation.

***Validation Methods* [10].** (i) *Linearity:* Linearity was obtained by plotting the five standard concentrations against absorbance. Each concentration was measured three times. The results are averaged, then made the equation (y = ax + b) and the correlation coefficient (r) with linear regression. (ii) *Limits of detection (LOD), and limits of quantitation (LOQ):* The LOD and LOQ were calculated by the following equations, i.e. LOD = 3 SD/slope and LOQ = 10 SD/slope, where SD is the standard deviation from linear curve. (iii) *Accuracy:* The recovery values were expressed the percentages for the ratio of the total phytosterol contents experimentally determined and their theoretical concentrations. Three standard concentrations, each concentration were measured three times, and the amount recovered was calculated. (iv) *Precision:* Six individual standard concentrations were examined for repeatability. Precision was expressed by the coefficient of variation (CV) = SD/average.

***Quantification of total phytosterol content.*** Five standard concentrations (25, 50, 75, 100 and 125 ppm), 1 mL of each concentration were added to 1 mL of the sample solution, 4 mL LB, and chloroform in 10 mL volumetric flask. The mixture was incubated for 10 min, then measured at 626 nm. Total phytosterols content was calculated by the standard addition method.

***Soy Yoghurt Characterization***

Organoleptic test, i.e. appearance, odor, taste, and consistency which carried out by 10 untrained panelists. The assessment criteria were (1) liked extremely, (2) liked slightly, (3) neutral, (4) disliked extremely, (5) disliked slightly [11]. The degree of acidity (pH) was measured with a calibrated pH meter. Total solids were calculated as the remaining sample weight after heating in an oven at 100 ± 1 °C for 4 hours [12].

## RESULTS AND DISCUSSION

### Phytochemical screening

Phytochemical screening results showed that soybean contain flavonoids, polyphenols, saponins, quinones, monoterpenoid, sesquiterpenoids, terpenoids, and phytosterols.

### Phytosterols Extraction

Soybean phytosterols were extracted by n-hexane and ethanol (82:18). n-hexane was chosen, because of good solubility of phytosterols, whereas ethanol can improve the extract organoleptic. After extraction, followed by saponification, then n-hexane phase was collected and vaporated. The yield was 8.58% (4.29 g of n-hexane extract). Phytosterols of soymilk and soy yoghurt were extracted by saponification, followed by liquid-liquid extraction. n-hexane extract was vaporated and the yield is calculated (Table 1). The highest yield was soy yoghurt with 6 h fermentation. The length of fermentation affect the non-polar secondary metabolites which dissolved in the n-hexane.

**Table 1. Phytosterols Yield**

| Sample | Yield (%) |
|---|---|
| Soymilk | 0.08 |
| Soy yoghurt 2 h | 0.10 |
| Soy yoghurt 4 h | 0.19 |
| Soy yoghurt 6 h | 0.20 |
| Soy yoghurt 8 h | 0.16 |
| Soy yoghurt 10 h | 0.05 |
| Soy yoghurt 12 h | 0.05 |

### Qualitative Analysis

The presence of phytosterols in the samples were confirmed by TLC. The chromatogram showed that all samples contain phytosterols. It is shown from the formation of blue-greenish spots with similar Rf as standard (Table 2).

**Table 2. TLC result**

| Sample | $R_f$ | Spot color with LB |
|---|---|---|
| Standard | 0.420 | blue-greenish |
| Soybean | 0.416 | blue-greenish |
| Soymilk | 0.416 | blue-greenish |
| Soy yoghurt 2 h | 0.416 | blue-greenish |
| Soy yoghurt 4 h | 0.416 | blue-greenish |
| Soy yoghurt 6 h | 0.416 | blue-greenish |
| Soy yoghurt 8 h | 0.416 | blue-greenish |
| Soy yoghurt 10 h | 0.416 | blue-greenish |
| Soy yoghurt 12 h | 0.416 | blue-greenish |

**Quantitative Analysis of Total Phytosterol Content**

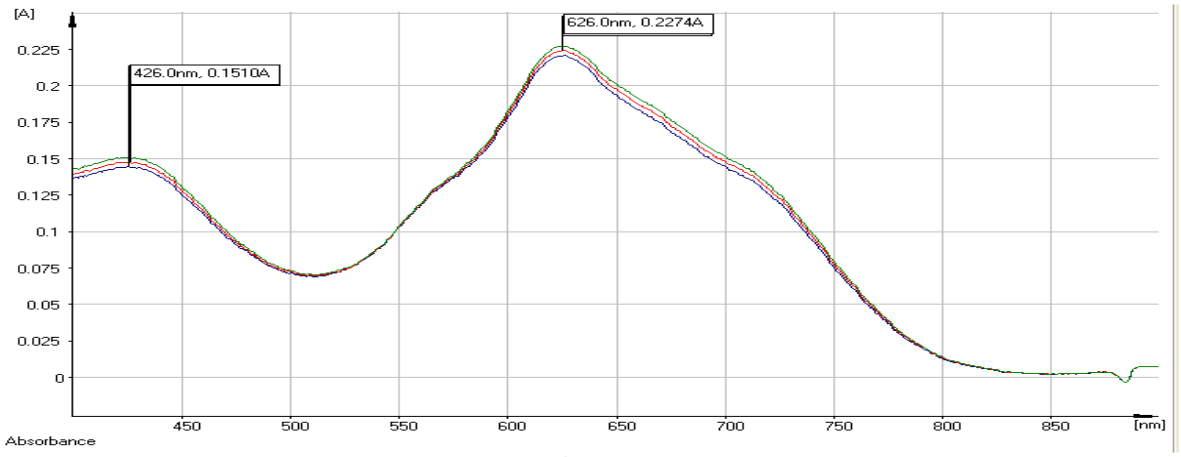


**Fig. 1. Spectrum for LB product reaction**

The reaction of LB was exothermic. The LB reactivity was reduced in long-term storage, so can not react with phytosterols. The first stage in the colorimetric reaction is formed of the protonated phytosterols by LB, followed by dehydration to form the carbonium ion of 3,5-cholestadiene. Next, the blue color is formed by an oxidation reaction, from pentacyclic cations [13]. The maximum wavelength was observed for the blue product at 626 nm (Fig. 1). One additional maximum wavelength can be observed at 426 nm from aromatic sulfonic acids, after being rearranged [14]. The appropriate wavelengths for analysis was 626 nm, because of accords with the literature, and is more specific to the reaction product from LB-phytosterols.

The maximum absorbance occurs 10 min after LB addition (Fig. 2). This confirms the accelerated behavior of the reaction [15], which is caused by the conversion of acetate derivatives of the steroids after the reaction with LB [13], where the instability product produces absorbance reduction.

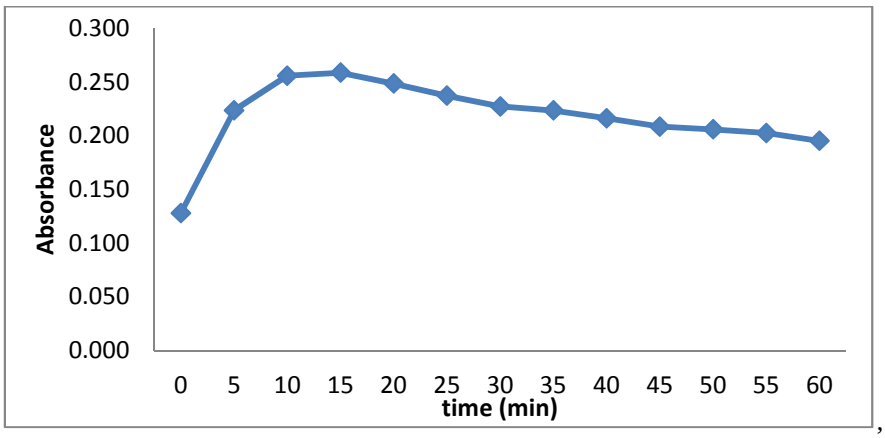


**Fig. 2. Influence of reaction time on absorbance**

Excess acid from LB resulted in decreased absorbance, which served after 5 mL LB was added (Fig. 3). It was because of instability products was increased and more degradable easily [13].

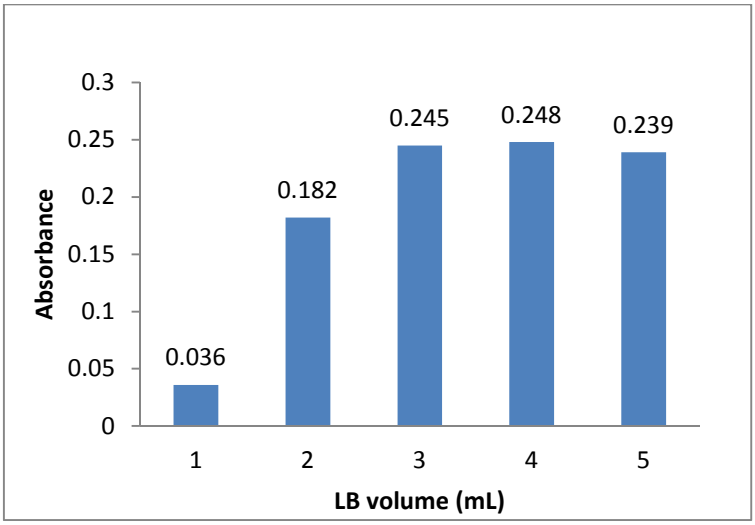


**Fig. 3. Influence of LB volume to absorbance**

**Validation Method**

Linearity shows the degree of correlation between the analyte concentration with detector response. There was a good correlation between the analyte concentration and detector response (r = 0.999, Fig. 4). This value accords with literature [10].

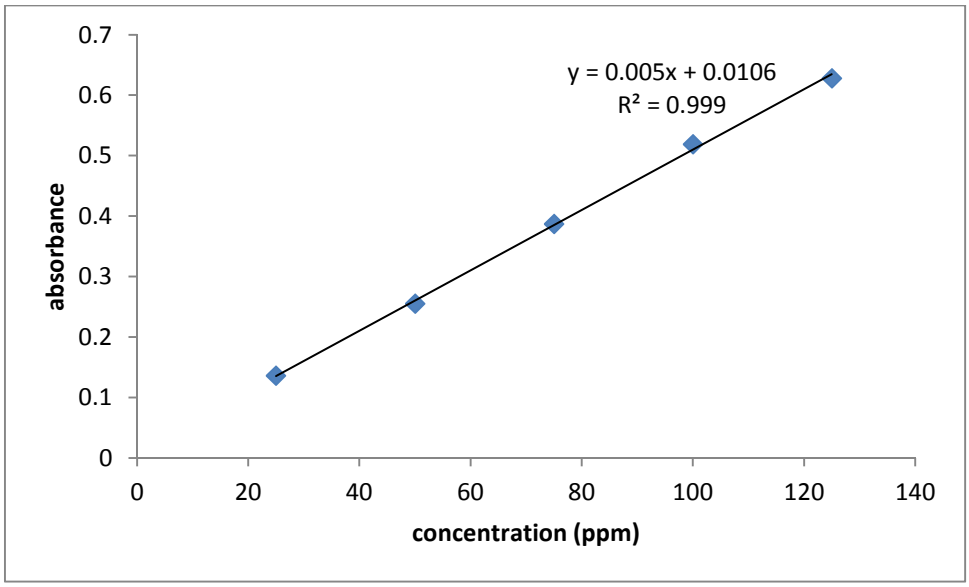


**Fig. 4. Linearity curve**

The LOD and LOQ was 0.162 ppm and 0.541 ppm, respectively. These values indicate the spectrophotometric procedure was sensitive for the detection and quantification of phytosterols. The accuracy and recovery expresses the nature of sample matrix. The procedures must be free from interference, and the responses should be due to the analyte. Table 3 showed that recoveries from 98.85 to 101.10%, accords with ICH, and provide reliable results [10]. Precision was assayed through repeatability. The 75 ppm standard was produced RSD of 1.23 and CV at 1.58. The method was considered precise according to ICH [10].

**Table 3. Accuracy result**

| Sample | Recovery (%) |
|---|---|
| 1 | 99.97 ± 1.79 |
| 2 | 101.10 ± 1.12 |
| 3 | 98.85 ± 1.15 |

**Quantification of Total Phytosterol Content**

**Table 4. Total Phytosterol Content in Sample**

| Sample | Total phytosterol content (ppm) |
|---|---|
| Soymilk | 27.84 |
| Soy yoghurt 2 h | 18.25 |
| Soy yoghurt 4 h | 54.34 |
| Soy yoghurt 6 h | 55.11 |
| Soy yoghurt 8 h | 102.86 |
| Soy yoghurt 10 h | 12.33 |
| Soy yoghurt 12 h | 19.54 |
| Soybean extract | 409.40 |

Fermentation with lactic acid bacteria will convert components and composition of bioactive compounds. Fermentation for 48 h can reduced the total phytosterol content significantly. This is due to the oxidation and dehydration [16]. The highest Total phytosterol content was soy yoghurt with 8 h fermentation. This result is contracdictive to extraction yield, which the highest yield is soy yoghurt with 6 h fermentation. This indicates that in the n-hexane extract contain non-polar compounds besides phytosterol. The total phytosterol content were decreased in soy yoghurt with 10 h and 12 h fermentation, because of phytosterols are unstable at acidic pH.

**Soy Yoghurt Characterization**

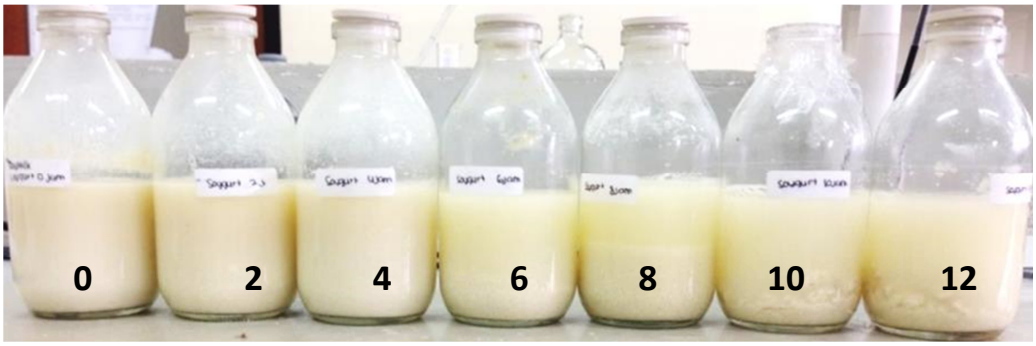


**Fig. 5. Soy yoghurt with variuos fermentation period**

The best organoleptic results is soy yoghurt with 4 h fermentation. This soy yoghurt was a viscous fluid with yellowish white color, specific odor, and tasted as fermented soymilk which meet the criteria [12]. Most volunteers (80%) was chosen the soy yoghurt with 4 h fermentation as the best soy yoghurt, because of the taste and the viscosity.

**Table 5. pH and total solid of soy yoghurt**

| Sample | pH | total solids (%) |
|---|---|---|
| Soy yoghurt 0 h | 7.0 | 14.9 |
| Soy yoghurt 2 h | 6.0 | 12.8 |
| Soy yoghurt 4 h | 5.5 | 13.6 |
| Soy yoghurt 6 h | 5.0 | 15.2 |
| Soy yoghurt 8 h | 4.5 | 18.8 |
| Soy yoghurt 10 h | 4.0 | 13.6 |
| Soy yoghurt 12 h | 3.5 | 10.3 |

The pH range for yoghurt is 3.8 to 4.6 [17]. More longer the fermentation period, the pH is more acidic, because of increasing the lactic acid content. Total solids was increased during fermentation from 2 h to 8 h, which propotional with lactid acid production [17]. It was due to the role of the lactic acid and lactase enzyme in the protein coagulation process, which causes an increasing total solids. However, total solids was decreased in soy yoghurt with 10 h and 12 h fermentation, because of the solids precipitation, which could not dissolved in n-hexane as the solvent in extraction. We choose the soy yoghurt with 4 h fermentation as the best soy yoghurt, based on the organoleptic, pH, total solids, and hedonic results. Although the soy yoghurt with 8 h fermentation have the highest total phytosterol content (102.86 ppm) compared to the soy yoghurt with 4 h fermentation (54.34 ppm).

## CONCLUSION

Colorimetric method is a valid procedure to determine the total phytosterol content in herbal derivatives.

## REFERENCES

[1] Trautwein, E.A. and I. Demonty, *Phytosterols: natural compounds with established and emerging health benefits.* Oilseeds and fats, Crops and Lipid, 2007. **14**(5): p. 259-66.
[2] Dutta, P.C., *Phytosterols as Functional Food Components and Nutraceuticals*2005, New York: Mercel Dekker Inc.
[3] Sanders, M.E., *Lactic acid bacteria as promotors of human health*, in *Functional Foods*, L. Goldberg, Editor 1997, Chapman and Hall: New York. p. 294-322.
[4] Osman, M.M.D. and K.A.A. Razig, *Quality Attributes of Soy-yoghurt During Storage Period.* Pakistan Journal of Nutrition, 2010. **9**(11): p. 1088-93
[5] Farnsworth, N.R., *Biological and Phytochemycal Screening of Plants.* J Pharm Sci, 1966. **55**: p. 225-86.
[6] Nelson, A.I., M.P. Steinberg, and L.S. Wei, *Illinois Process for Preparation of Soymilk* J. Food Sci., 1976. **41** p. 57-61.
[7] Kanda, H., et al., *Yoghurt production by Lactobacillus fermentation of soybean milk.* Proc. Bichem., 1976: p. 23-25.
[8] Ham, B., B. Butler, and P. Thionville, *Evaluating the Isolation an Quantification of Sterols in Seed oils by Solid phase extraction and capillary gas liquid chromatography.* LCGC 2000. **18** (11 ): p. 1174-81.
[9] Goad, L.J. and A. Toshihiro, *Analysis of Sterol*1997, London: Blackie Academic & Propesional.
[10] EMA, *ICH Topic Q 2 (R1), Validation of Analytical Procedures: Text and Methodology*, in *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*2006, European Medicines Agency: London.
[11] Stone, H. and J.L. Sidel, *Sensory Evaluation Practices*2004, St Louis, Missouri, USA: Academic Press.
[12] AOAC, *Official method of analysis of Association of Official analytical Chemist*. 18 ed2005, Washington D C: Association of Official Analytical Chemists.
[13] Xiong, Q., W.K. Wilson, and J. Pang, *The Liebermann-Burchard reaction: sulfonation, desaturation, and rearrangment of cholesterol in acid.* Lipids, 2007. **42**: p. 87-96.
[14] Burke, R.W., et al., *Mechanisms of the Liebermann-Burchard and Zak color reactions for cholesterol.* Clin. Chem., 1974. **20**: p. 781-94.
[15] Moore, P.R. and C.A. Baumann, *Skin sterols. I. Colorimetric determination of cholesterol and other sterols in skin.* J. Biol. Chem, 1952. **195**: p. 615-21.
[16] a, J.H., et al., *Effects of fermentation on the phytochemical composition and antioxidant properties of soy germ* Food Chemistry, 2008). **109**: p. 709-21.
[17] Tamime, A.Y. and R.K. Robinson, *Yoghurt: Science and Technology*. 2 ed. CRC Press1999, Boca Raton, FL.