ISSN: 2356-7767

# Validated method for extraction and purification of Lipopolysaccharide from Escherichia coli and Salmonella by HPLC

**Mai, A. Fadel[*] and Heba, M. Hassan[**]**

[*]Pharmacology and Pyrogen Unit, Chemistry, toxicology and Food Deficiency Department, Animal Health Research Institute, Dokki, P.O. Box 246, Giza 12618, Egypt

[**]Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, P.O. Box 246, Giza 12618, Egypt

***Corresponding author:** Mai A Fadel

E-mail: dr.mai87@yahoo.com

Received in 1/10/2019
Accepted in 4/11/2019

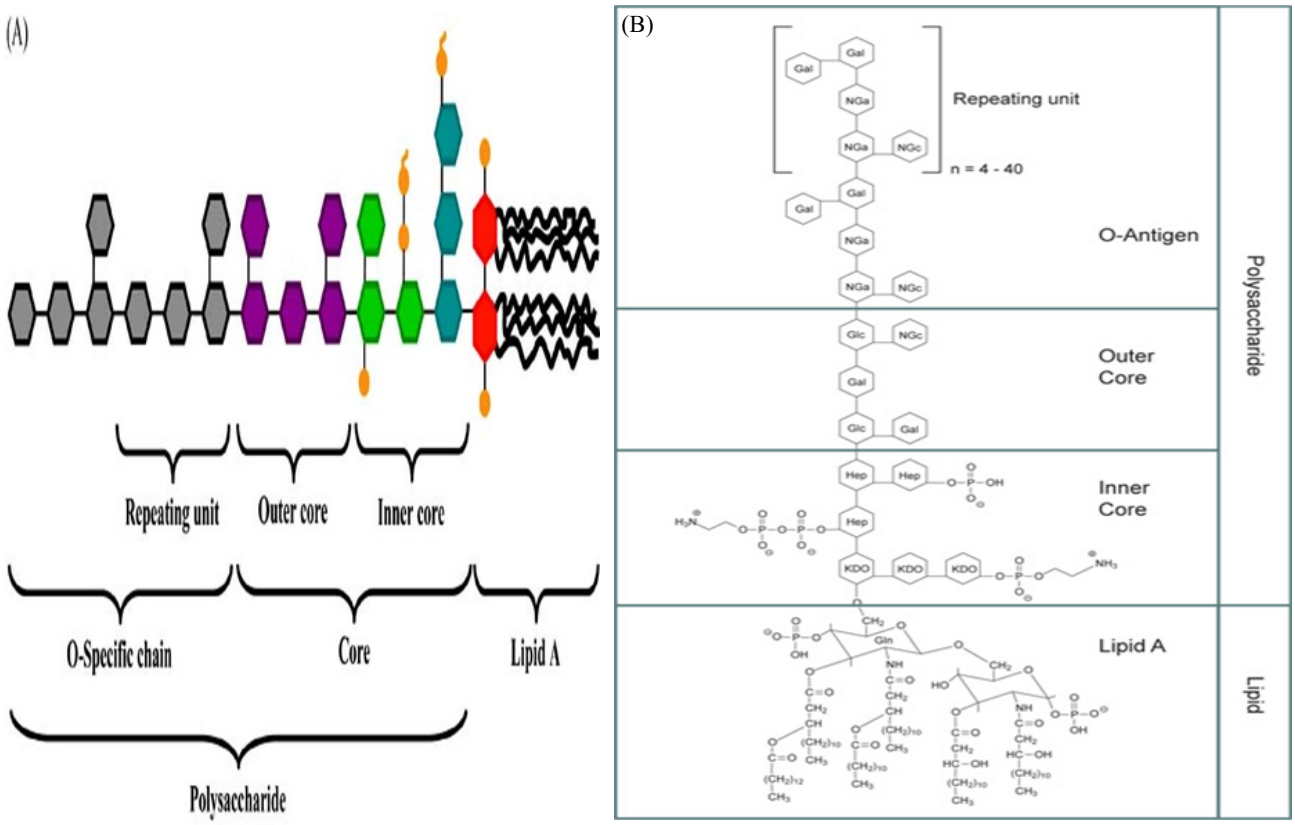
**Abstract**

Most methods used for extraction and purification of Lipopolysaccharide (LPS) were time-consuming and with an excessive number of chemicals so that very expensive. LPS were obtained from Gram-negative bacteria as *Escherichia coli* and *Salmonella* LPS in gram-negative bacteria has the complex construction and is composed of three parts including the complex part named fat A, polysaccharide, and O side chain. The current study declared a simple, precise, rapid and accurate developed method for extraction and detection of LPS by High-Performance Liquid chromatography (HPLC). The extraction procedures were carried out by isopropanol and sodium hydroxide and the chromatographic separation was performed with a reversed-phase column (Dionex Acclaim TM 120, C18 (150 × 4.6 mm, 5 μm) with a mixture of water and methanol (65:35) as mobile phase with best UV detection at 210 nm. The extracted LPS were calibrated on the standard curve of LPS reference standard with a linearity range (0.005-1μg/ml) within a correlation coefficient ($r^2 = 0.9999$). The limits of detection (LOD) and quantification (LOQ) were 0.003597μg/ ml and 0.010899μg/ ml respectively. The analytical conditions gave a good recovery in a range of less than 2%.

***Keywords:*** *Lipopolysaccharide, HPLC, Escherichia coli, Salmonella.*

## Introduction

LPS are amphiphilic molecules, composed of a lipid region covalently bound to a polysaccharide moiety by an ester bond. The latter includes the specific O side chain and the central oligosaccharide, which contains 2-keto-3-deoxyoctulosonic acid (KDO). Lipid and polysaccharides are linked through KDO groups. The lipid component is composed of the disaccharide glucosamine, which is bound to fatty acid chains (from four to six) by ester and amide bonds. **Brandenburg and Wiese, (2004).**




(A) Chemical structure of Lipopolysaccharide (LPS), and (B) General structure for bacterial lipopolysaccharides. Abbreviations: KDO: 3-deoxy-α-D-mannooctulosonic acid; Hep: Heptulose (ketoheptose); NGa: Galactosamine; NGc: Glucosamine. The color represented in (A) denotes different sugar moieties.

The lipopolysaccharide component of the cell surface of gram-negative bacteria has been shown to have a multiplicity of biological roles and activities. Its functions within the bacterium include a role in the selective permeability barrier of the cell **(Leive *et al.,* 1968 and Voll and Leive, 1970)** and as a receptor for adsorption of some bacteriophages **Rapin and Kalckar, (1971).** Interactions of LPS with other biological systems include toxicity to many higher organisms, immunogenicity, and activation of serum complement **(Landy and Braun, 1964**, **Morrison and Lieve, 1975**). LPS recognized by immune cells as a pathogen-associated molecule. They can cause severe diseases like sepsis and therefore known as endotoxins **Wang *et al.,* (2010**). Enzyme-linked immunosorbent assay (ELISA) using *Salmonella* lipopolysaccharide (LPS) to measure specific IgG titers as serology can be used to assess vaccine responses and infection rates, to detect carriers, and to aid in epidemiologic studies **Smith *et al.,* (1995)** and through their study proved that *Salmonella* serology using LPS antigens is highly O antigen-specific and predictable.

Endotoxins or lipopolysaccharides (LPS) from Gram-negative bacteria can be extracted by several methods such as trichloroacetic acid extraction at 4ºC **Staub, (1965),** aqueous butanol **(Morrison and Lieve, 1975),** triton/ Mg+2 **(Delahooke *et al.,* 1995),** cold ethanol **(Sonesson *et al.,* 1989)** and extraction in water at 100ºC **(Eidhin and Mounton, 1993)**. Other extraction methods with phenol, chloroform, petroleum-ether **Galanos *et al.,* (1969)** and methanol **(Nurminen and Vaara, 1996)** have been described specifically for rough LPS (R-LPS).

The purpose of this work is to produce a high-

ly sensitive, accurate, reproducible, rapid, safer and precise validated method for purification of LPS from gram-negative bacteria like *E. coli* and *Salmonella*.

## Materials and Methods

**Samples collection:**

*Salmonella* and *E. coli* isolates were collected from apparently diseased chickens; Samples from internal organs such as (liver, spleen, heart, and intestine) were collected aseptically then labeled and transported directly in the ice box to reference laboratory for Veterinary Quality Control on poultry production for further examinations as soon as possible

**Bacterial isolation and identification:**

***Salmonella* isolation, identification and serotyping.**

*Salmonella* was isolated and identified from the collected internal organs according to **ISO 6579-1 (2017)**. All isolates that were biochemically identified as *Salmonella* were then subjected to serological identifications according to **Le Minor and Popoff, (1987).**

***E. coli* isolation, identification:**

All samples were examined bacteriologically for presence of *E. coli*. Isolation and identification of *E. coli* was done according to **Lee and Arp** (**1998**) and **Quinn *et al.,* (2002)** and serotyped by Denka Seiken Co. antisera.

All the collected samples were pre-enriched in buffered peptone water (Oxoid) and incubated at 37°C for 24 hrs under aerobic conditions.

Then a loopful from each broth culture was inoculated onto blood agar, MacConkey agar (Oxiod), XLD agar (Oxiod) and Eosin methylene blue agar plates and incubated at 37ºC for 24 hours. Isolated colonies were identified morphologically, microscopically and biochemically

**Chemical and reagents:**

All used chemicals during extraction and analysis were analytical grade. Methanol and water (Fischer) were HPLC grade. Extra pure *salmonella typhymurium* LPS (Sigma, Saint Louis, USA) was used as a standard

**Instrumentation**

(HPLC) system was used. HPLC compartments were Agilent Series 1200 quaternary gradient pump, Series 1200 autosampler, Series 1200 UV detector, and HPLC 2D Chemstation software (Hewlett-Packard, Les Ulis, Germany).

**Standard preparation and the chromatographic separation:**

Stock standard solution was prepared by dissolving 1 mg of lyophilized (LPS) in deionized water (1 ml) and dilute to have different concentrations (0.005, 0.01, 0.05, 0.1, 0.2, 0.4, 0.8 and 1µg/ml) for linear equation. The best peak resolution was at 3.455 min. as a retention time (RT). The flow rate was 1ml/min and multi-wave detector at a wavelength of 210 nm. The analyte was injected on HPLC with an injection volume 10 µl and the column temperature was ambient.

**Extraction procedures**

*Salmonella* and *E. coli* cells suspended in peptone water were centrifuged at 10000 g for 5 min. The pellets were collected and washed twice by PBS (pH 7.2) and discard the supernatant. The pellets (10 mg) were weighed and suspended in 0.5 ml of a mixture consisted of propanol: sodium hydroxide 1 mol/ ml (5:3 v:v) with alkaline pH 11.4 and were kept in 100ºC water bath in tightly closed Eppendorf for 2 hours with gentle mixing by a magnetic stirrer. The mixture was cooled in Freezer at -20ºC and ultra-centrifuged at 10000 g for 15min. The supernatant was collected and the sedimented gel-like layer was extracted by 312.5µg isopropanol and ultra-centrifuged at 10000 g for 5min. The precipitate was discarded and the supernatant was added to the previous one and diluted by an equal volume of distilled water. The sample was centrifuged and supernatant was collected. The insoluble LPS were extracted by 100 µl 35% methanol in cold water for 20 minutes with a magnetic stirrer at 30 rpm. The sample was centrifuged at 2000 rpm for 5min at $4^0$ C. The supernatant was collected and injected on HPLC.

**Method validation**: United States Pharmacopeia (USP) and International Conference for Harmonization (ICH) guidelines are followed for the validation of the proposed method, in terms of system suitability, specificity, (linearity and Range), (Precision and accuracy), Limit of detection (LOD) and Limit of quantification (LOQ), and robustness.

**System suitability:**

System suitability test (SST) is a test to determine the suitability and effectiveness of the

chromatographic system before use. SST was determined by characteristic chromatographic parameters like Resolution (Rs), Tailing factor (Tf), peak asymmetry factor (As) and the number of effective theoretical plates (N).

**Specificity:**
Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

**Linearity and Range:**
A linear relationship should be evaluated across the range of the analytical procedure. Linearity was performed by preparing different concentrations of LPS standard at a squared correlation coefficient of 0.99 ($r^2$) according to ICH.

**Precision and accuracy:**
Precision is the closeness of agreement among a set of results it included intra-day precision (Repeatability) and inter-day precision (within -laboratory variation). Intraday precision evaluated by six injections of the same concentration in the same day but inter-day precision throughout six days of the week. The accuracy of the assay was assessed by comparing the calculated mean concentrations to the actual concentrations of serial dilutions. The accuracy was required to not exceed 1%, and the intraday- and inter-day precisions were not to exceed 1%.

**(LOD) and (LOQ):**
It is considered to be the quantity yielding a detector response which gives the signal to noise ratio 3:1 according to (ICH). Whereas LOQ is the lowest amount that can be analyzed within acceptable precision and accuracy which give the signal to noise ratio 10: 1 according to (ICH).

**Robustness:**
The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

**Results**
**Bacterial isolation and identification:**
Eight *Salmonella* isolates were isolated from 100 diseased broiler chickens in Dakahlia Governorate with an incidence of (8%). Two *S. typhimurium* were identified serologically
Our results revealed that, the overall prevalence of *E. coli* in all examined samples was 18% (18/100) and confirmed serologically

**Method validation**
The chromatographic separation was demonstrated through the following characteristics:

**System suitability test:**
According to USP, 2017, column theoretical plates (N) =≥2000, Peak asymmetry (As) is ≥1 and Tailing factor (T) should be= ≤1.0. In the present study, theoretical plates (N) were 4755.333. Peak asymmetry factor (As) was 0.963 and T was 1.00.

**Specificity:**
The chromatograms compared to know that there is no excipient compound interference between peaks of the pure standard Fig. (2) and the extracted LPS from gram-negative bacteria (*Salmonella* and *E. coli*) and other impurities illustrated in **Fig. (3) and Fig. (4)** and the retention time of the standard and sample peak was 3.455min..

**Linearity and Range:**
Standard curves were constructed by dilution of LPS standard stock solution with various volumes to yield a concentration range of 0.005, 0.01, 0.05, 0.1, 0.2, 0.4, 0.8 and 1 µg/ ml. Linearity existed within a range of 0.005 and 1 µg /ml. The linearity of peak area responses versus concentrations was demonstrated by linear least square regression analysis. The linear regression equations were Y = 692.04x - 0.1747 with a correlation coefficient ($r^2$ = 0.9999) **Fig. (1).**

**Precision and accuracy:**
The method for separating LPS was precise as the RSD for both intra-day precision and inter -day precision as shown in table (1).

**Table (1).** Intra and Inter-day precision at concentration 0.05 µg/ml.

| Ser. no. | Concentration level (µg/ml) | Intraday precision | Inter-day precision |
|---|---|---|---|
| | | Peak area | |
| **1** | 0.05 | 34.853 | 34.087 |
| **2** | 0.05 | 34.782 | 33.921 |
| **3** | 0.05 | 34.806 | 33.8889 |
| **4** | 0.05 | 34.337 | 34.674 |
| **5** | 0.05 | 34.591 | 34.364 |
| **6** | 0.05 | 34.645 | 33.987 |
| **Mean** | | 34.669 | 34.15365 |
| **SD** | | 0.190933 | 0.307065 |
| **RSD%** | | 0.550733 | 0.899069 |

The LPS standard was added to known sample quantity (0.06µg/ml) which from 10 mg of extracted bacterial pellets as shown in table (2) with triplicate readings. The accuracy studies were confirmed by adding the known sample quantity (0.06µg/ml) to different dilution of LPS standard to have the final three concentration levels 0.1, 0.2 and 0.4µg/ml table (3). The method was accurate according to the calculated test results from the % recovery which ranged from 99.899-100.13%.

**Table (2).** Recovery studies for extracted LPS sample.

| Sample concentration (0.06 µg/ml) with triplicate readings | Mean±SD | Recovery% |
|---|---|---|
| **0.06058**<br>**0.06013 (*salmonella* extract sample)**<br>**0.06029** | 0.060336±0.00023 | 100.5603 |
| **0.05892**<br>**0.05894 (*E. coli* extract sample)**<br>**0.05955** | 0.059133±0.00036 | 98.55427 |

**Table (3).** Accuracy studies for *Salmonella* and *Escherichia coli* extract sample.

| Actual conc.in extracted LPS sample | Added conc. at 3 levels | Resulted conc. levels | Found conc. | Mean± SD | RSD% | Recovery% | Average re-covery%±SD (Accuracy) |
|---|---|---|---|---|---|---|---|
| **0.060336** ***(Salmonella)*** | 0.04 | 0.1 | 0.100419<br>0.100039<br>0.099943 | 0.100133 ± 0.000252 | 0.251281 | 100.4186<br>100.0386<br>99.94292 | 100.1334± 0.251616 |
| | 0.140 | 0.2 | 0.199696<br>0.200609<br>0.199995 | 0.2001 ± 0.000466 | 0.232677 | 99.84806<br>100.3047<br>99.99762 | 100.0501± 0.232794 |
| | 0.340 | 0.4 | 0.399796<br>0.399556<br>0.39944 | 0.399597 ± 0.000181 | 0.045376 | 99.94896<br>99.88899<br>99.86009 | 99.89934± 0.04533 |
| **0.059133** ***(E. coli)*** | 0.04 | 0.1 | 0.099163<br>0.099056<br>0.098928 | 0.099049 ± 0.000117 | 0.118616 | 99.16291<br>99.05598<br>98.92824 | 99.04904± 0.117488 |
| | 0.140 | 0.2 | 0.197543<br>0.1983<br>0.19968 | 0.198508 ± 0.001084 | 0.545842 | 98.77168<br>99.14976<br>99.84011 | 99.25385± 0.54177 |
| | 0.340 | 0.4 | 0.395412<br>0.397456<br>0.394496 | 0.395791 ± 0.00152 | 0.384131 | 98.85292<br>99.36626<br>98.62403 | 98.94774± 0.380089 |

**Limit of detection (LOD) and Limit of quantification (LOQ)**
LOD and LOQ were calculated according to ICH guidelines according to the following equations:
LOD= 3.3 × σ/S
LOQ = 10 × σ/S
Where σ is the standard deviation of intercept S is the slope of the calibration curve. LOD of LPS standard was 0.003597µg/ mL and LOQ was 0.010899µg/ ml.

**Robustness:**

**Table (4).** The applied robustness conditions at concentration level 0.2 µg/ml of LPS standard.

| Robustness parameter | Condition checked |
|---|---|
| **Detection wavelength** | 210, 208, and 212 nm |
| **The flow rate of the mobile phase** | 0.8, 1.0, and 1.2 mL/min |
| **Mobile phase composition (Methanol: HPLC water)** | 35:65<br>37:63<br>33:67 |
| **Analyst days** | Two analysts analyzed the same trial on the same day<br>The same analyst analyzed the same trial in 2 different days |

The results demonstrated that the developed method was robust and reproducible (ruggedness) at the mentioned conditions stated in Table (4). All the results of the analyzed samples were consistent, and the RSD of all the tests at different conditions was less than 2.

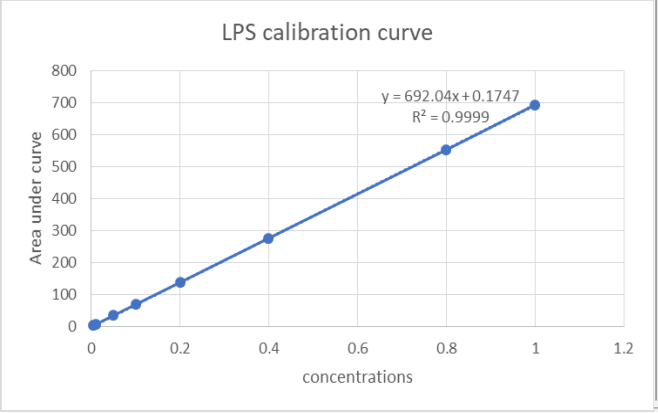



**Fig. (2):** LPS standard curve

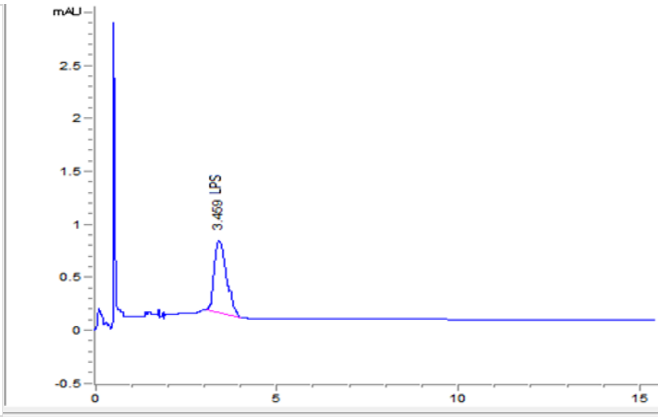



**Fig. (2):** HPLC Chromatogram of LPS of *Salmonella typhimurium* concentration standard (0.005 µg/ml).

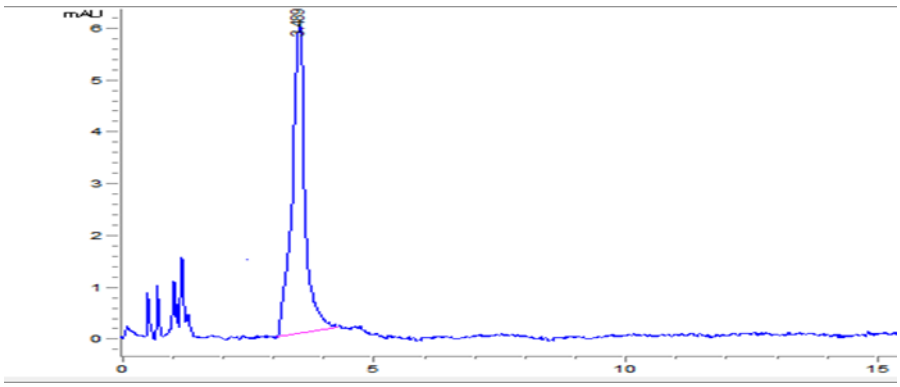



**Fig. (3):** HPLC Chromatogram of extracted LPS from *Salmonella typhimurium* with concentration (0.06 µg/ml).

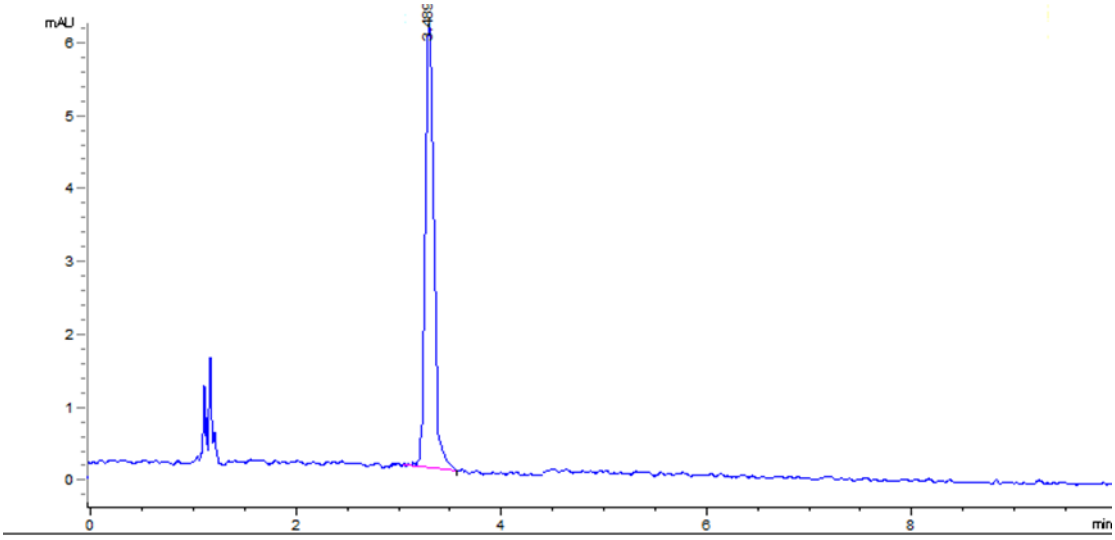



**Fig. (4):** HPLC Chromatogram of extracted LPS from *E.coli O157* with concentration (0.06µg/ml).

## Discussion

The present proposed method was depended on extraction of LPS from gram-negative bacteria as salmonella and *E. coli* by using chemicals of alcoholic nature. The first step was done for precipitation of bacterial protein and extraction of LPS by iso-propanol, NaoH mixture and the supernatant contained LPS and nucleic acid as **Westphal, (1951)** who used phenol-water mixture for the same purpose. Ultracentrifugation aimed to the separation of nucleic acids and LPS which precipitated, this was approved by **Tauber and Garson, (1959).** Isopropanol and methanol yielded more LPS extract as confirmed by **Perdomo and Montero, (2006)** as using of alcohols precipitated more LPS. Nevertheless, it has never been characterized as to why NaOH was chosen as the best chemical to produce LPS with free DNA content. In addition, the question arises as to whether the chemically-induced bacteria as *E. coli* maintain LPS on their cell envelope in the same way as untreated wild-type bacterial cells, because alkaline hydrolysis is known as a depyrogenation method that destroys the 8 carbon sugar: 2-keto-3-deoxyoctonic acid that links Lipid-A to the core polysaccharide **Sandle, (2011).**

On the other hand, it has been described that the poly-saccharide moiety and the lipid A fatty acids, are capable of absorbing at wavelengths near 200nm. This was concluded from study of **Seid and Sadoff**, **(1981)** who demonstrated that fatty acid lacking LPS do absorb

UV near 200nm. The same observation was studied by **Volpi, (2003)** for detoxified LPS (lipid A free) and **Pier *et al.,* (1978)** for bacterial polysaccharides.

Herein, several wavelength trials were done to have the best wavelength for detection of LPS, and the clearest detectable and quantifiable peak was at 210 nm.

In this work-study, we used high temperature (100°C) and alkaline conditions (pH 11.4) for complete removal of resistant proteins, which is compatible with result of **Eugene and Hackett, (2000).**

The advantages of this research over other published research are that study used simpler method; for example, **Rezania *et al.,* (2011)** used long run time as the retention time in their work was 25 minutes, whereas, in our case, the time of run has been reduced from 25 to 3.4 minutes. Moreover, in our work, we depended on lesser use of extraction reagents which is more economic unlike other methods as **Morrison and Lieve, (1975)** who used phenol method versus butanol extraction and **Mirzaei *et al.,* (2011)** who introduced methanol-chloroform extraction method with electrophoresis with use more poisonous chemicals like bis acrylamide which is neurotoxic.

## Conclusion

There are different methods for extraction, separation, and purification of LPS which are costly and toxic. Herein, we introduced a simple, rapid, economical, sensitive, precise and safer method. The obtained results were precise, accurate, and sensitive. The developed analytical procedure can be adapted by international pharmacopoeias and can be used by quality control labs.

## References

**Brandenburg, K. and Wiese, A. (2004).** Endotoxins: relationships between structure, function, and activity. Curr. Topics. Med. Chem; 4(00): 1-20.

**Delahooke, D.M.; Barclay, G.R. and Poxton, I.R. (1995).** A repraisal of the biological activity of bacteroides LPS. J. Med. Microbiol; 42(2): 102-12.

**Eidhin, D.N. and Mounton, C. (1993).** A rapid method for preparation of rough and smooth lipopolysaccharide from bacteroides, Porphyromonas and Prevotella. FEMS Microbiol Lett; 110(2): 133-8.

**Eugene, C.Y. and Hackett, M. (2000).** Rapid isolation method for lipopolysaccharide and lipid A from gram-negative bacteria. Analyst, 125(4), 651-656.

**Galanos, C.; Luderitz, O. and Westphal, O. (1969).** A new method for the extraction of R lipopolysaccharide. Eur. J. Biochem; 9(2): 245-9.

**ISO. 6579-1 (2017).** Microbiology of the food chain – horizontal method for the detection, enumeration and serotyping of Salmonella – part 1: detection of Salmonella spp. 1st ed.

**Landy, M. and Braun, W. eds (1964).** Bacterial Endotoxins, Quinn and Boden Co. Inc. Rahwav. N. J.

**Lee, M.D. and Arp, L.H. (1998).** A laboratory manual for the isolation and identification of avian pathogen Daviv E. Swayne, Chairman, John R. Glisson, Mark W. Jack wood, James E. Pearson, Willie M. Reed. Editorial Board for the American Association of Avian Pathologists, 4th ed., Chapter 3, Colibacillosis.14-16.

**Leive, L.; Shovlin, V.K. and Mergenhagen, S.E. (1968).** J. Biol. Chem. 243, 6384-6391.

**Le Minor, L. and Popoff, M.Y. (1987).** Request for an opinion. Designation of *Salmonella enterica* sp. nov., nom. rev., as the type and only species of the genus *Salmonella*. Int J Syst Bacteriol. 37: 465–468.

**Morrison, D.C. and Leive, L. (1975).** Fractions of lipopolysaccharide from *Escherichia coli* O111:B4 prepared by two extraction procedures. J Biol Chem; 250(8): 2911-9.

**Mirzaei, A.; Ashtiani, M.H.H.R.A.; Rahbar, M. and Rastegar, H. (2011).** A simple method for non phenolic extraction of lipopolysaccharide from *Salmonella typhimurium* and *Salmonella* enteritidis with high purity and pyrogenicity in rat. Scientific Research and Essays, 6(5), 1101-1105.

**Nikaido, H. (1973).** In Bacterial Membranes and Walls (LEIVE,L., ed) pp. 131-208, Marcel Dekker, New York.

**Nurminen, M. and Vaara, M. (1996).** Methanol extracts LPS from deep rough bacteria. Biochem Biophys Res Commun; 219(2): 441 -2.

**Perdomo, R. and Montero, V. (2006).** Purification of *E. coli* 055: B5 lipopolysaccharides by size exclusion chromatography. Biotecnologia aplicada, 23(2), 124-129.

**Pier, G.B.; Sideberry, H.F.; Zolyomi, S. and Sadoff, J.C. (1978).** Isolation and characterization of high-molecular-weight polysaccharide from the slime of pseudomonas aeruginosa. Infect and Immun; 22(3): 908-18.

**Quinn, P.J.; Carter, M.E.; Markey, B.K.; Donnoly, W.J. and Leonard, F.C. (2002).** Veterinary microbiology and microbial disease.166-1117 Osney Mead, Oxford first LTd, Registered at the United Kingdom.

**Rapin, A.M.C. and Kalckar, H.M. (1971).** In Microbial Toxins (WEINBAUM, G., KADIS, S., and AJL, S. J., eds) Vol. 4, pp. 267-303, Academic Press, New York.

**Rezania, A.; Riedel, M.J.; Wideman, R.D.; Karanu, F.; Ao, Z.; Warnock, G.L. and Kieffer, T.J. (2011).** Production of functional glucagon-secreting α-cells from human embryonic stem cells. 60(1): 239-47.

**Staub, A.M. (1965).** Bacterial lipido-proteino polysaccharides (“O” somatic antigens). Extraction with tricloroacetic acid. In Whistler RL, Wolfan ML editors. Methods in Carbohydrate chemistry. New York: Academic Press Inc, (5): 92-3.

**Sandle, T. (2011).** A paractical approach to depyrogenation studies using bacterial endotoxin. J. GXP Compliance; 15: 90–96.

**Seid, R.C. and Sadoff, J.C. (1981).** Preparation andcharacterization of detoxified lipopoly-saccharide-protein conjugates. J. Biol-Chem; 256(14): 7305-10.

**Sonesson, A.; Jantzen, E.; Bryn, K.; Larsson, L. and Eng, J. (1989).** Chemical Composition of Lipopolysaccharide from *Legionella pnumophila.* Arch Microbiol 1989; 153: 72-8.

**Tauber, H. and Garson, W. (1959).** Isolation of lipopolysaccharide endotoxin. J. Biol Chem, 234(6), 1391-1393.

**Voll, M.J. and Leive, L. (1970).** J. Biol. Chem. 246, 1108-1114.

**Volpi, N. (2003).** Separation of *Escherichia coli* 055:B5 lipopolysaccharide and detoxified lipopolysaccharide by high-performance capillary electrophoresis. Electrophoresis; 24: 3097-103.

**Wang, X.; Zhang, C.; Shi, F. and Hu, X. (2010).** Purification and characterization of lipopolysaccharides. In Endotoxins: Structure, Function and Recognition (pp. 27-51).

**Westphal, O. (1951).** Bacterial irritants and analysis of their effects, especially on pituitaryadrenal cortex system. Praxis, 40(38), 789..