

THE USE OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN SEPARATION AND PURIFICATION OF LP 64 KDA BAND OF MYCOPLASMA GALLISEPTICUM IN LARGE QUANTITIES

EL-SHATER S. A. A., S. I. EISSA and A. M. HASSAN

Animal Health Research Institute, Agricultural Research Center, Dokki, Egypt.

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SUMMARY

High performance liquid chromatography (HPLC) size exclusion technique (SEC) was used for separation and purification of the LP 64 kDa band of *Mycoplasma gallisepticum* (MG) in large quantities. We found that the use of 210 nm was the best wave length for isolation and gave highest peak and the best resolution. The use of 200 mM of NaH_2PO_4 as a mobile phase improved chromatographic profile. Injection volume of 200 μl resulted in the highest peak and the best resolution. The flow rate of 5ml /min increased the peak height and profile resolution. The use of pH 5.7 for the *Mycoplasma gallisepticum* culture also improved the height of the chromatographic peak and the profile of resolution and was the optimum pH for production and purification. The use of HPLC-SEC could be adapted to extract and purify the intended LP64 kDa band of MG.

INTRODUCTION

Mycoplasma gallisepticum is one of the most important disease agents affecting poultry causing high losses to poultry farms. The LP64 kDa is a lipoprotein playing major role in cytoadherence of *Mycoplasma gallisepticum* (Forsyth et al., 1992). Many methods for isolation and purification of this lipoprotein were used such as pH of the medium, buffer and salts, detergents and chelators or metal ions but the most accurate is high performance liquid chromatography (HPLC) (Jansen and Rydén, 1998). Size-exclusion chromatography (SEC) has widely been used for separation and purification of proteins. SEC is an entropically controlled separation technique that depends on the relative size of macromolecules with respect to the size and shape of the pores of the packing. High performance size-exclusion chromatography (HP-SEC) columns are favored because of their speed, high resolution and high sensitivity (Laing

et al., 2001). Optimization of HP-SEC conditions such as wave length of the detection, ionic strength of the mobile phase, sample injection volume, flow rate and pH of the MG culture (Engelhardt and Ahr, 1983). Our work was to optimize the HPLC-size exclusion technique for production and purification of the LP 64 kDa band of *Mycoplasma gallisepticum*.

MATERIALS AND METHODS

Materials:

A Model P 580 pump and Model UVD 170 S detector were used (Dionex, USA). The column employed for separation by size exclusion (SEC) was GF-250X1 column 21.2 mm ID X 250 mm (Zorbax). The fraction collector was Foxy Jr (Isco, Inc., USA).

Methods:

1- Preparation of media:

The basal broth dehydrated culture media was added to 100 ml distilled water, then sterilized by autoclaving at 121°C for 15 min. After cooling to 50°C other enrichment ingredients; horse serum, yeast extract, DNA and inhibitors (Thallium and penicillin G sodium), were added aseptically. Sterility was checked by incubation of the media at 37°C over night then stored at 4°C for use within 2-3 weeks (Frey et al., 1968)

2- Culture passage

M. gallisepticum, strains F (vaccinal strain), R, PG31 (ATCC type strain) and A5969 (high -

passage chicken isolate), were grown in Frey's medium with 12% swine serum. All cultures were grown at 37°C and harvested by centrifugation at 12000x g for 20 min.

3-Integral membrane proteins extraction

Extraction of integral membrane protein was accomplished with triton X-114 by the method of (Bricker et al, 1988), with some modifications briefly pellets of *M. gallisepticum* (1.25 mg protein/pellet) were resuspended in 1 ml of ice cold. 1.0% (v/v) Triton X-114 was added in (10mM Tris, 150 mM NaCl buffer, pH 7.49 (TS buffer) with 2mM phenylmethylsulphonyl fluoride (PMSF, Sigma chemical co.) these were incubated in at 4°C for 30 min. Insoluble materials was pelleted by two cycles of centrifugation at 4°C for 15 min. at 13000 g. Supernatants were incubated at 37°C for 8 min. and centrifuged at 10000 g for 5 min at room temperature. The aqueous phase was readjusted with 10% Triton X-114 to 1.0% (v/v). The detergent phase was brought to the original volume with TS buffer. The previous method was repeated five times and the detergent phase was used for HPLC after dialyzing against three changes of 150 mM phosphate buffer saline (PBS) and stored at - 20°C until used.

4- Optimization of HPLC-SEC conditions:

4-1-Standard protein

64 kDa protein was isolated by electroelution after SDS-PAGE electrophoresis and then was used as standard protein for the separation by HPLC-

SEC procedure. The retention time of the protein was determined (from 6-11 min.) and 3 fractions could be separated having the same retention time.

4-2-Optimization of the wavelength.

Different wavelength 210 nm, 220 nm, 240 nm and 280 nm were used to optimize the wavelength, the column employed for separation by size exclusion was GF-250Xl column 21.2 mm ID X 250 mm (Zorbax). The mobile phase is 0.2 M di-sodium phosphate, pH 7.0.

4-3-Optimization of the strength of mobile phase.

Two mobile phase 100 mM and 200 mM NaH_2PO_4 were used to improve the chromatographic profile of separated protein, size exclusion GF-250Xl column 21.2 mm ID X 250 mm (Zorbax) was used. The injected sample was 200 μl , the flow rate 5 ml / min. and detection was by UV absorption at 210 nm.

4-4-Optimization of the sample injection volume.

Different volumes of the protein samples (20 , 40, 200 and 300 μl) were injected ,the column employed for separation by size exclusion was GF-250Xl column 21.2 mm ID X 250 mm (Zorbax).The mobile phase is 0.2 M disodium phosphate, pH 7.0 . The flow rate 5 ml / min. and de-

tection was by UV absorption at 210 nm. The fraction collector was Foxy Jr (Isco,Inc., USA).

4-5-Optimization of the flow rate.

Different flow rates (3 , 5 and 15ml/min) were used to improve the chromatographic profile of separated protein, the column employed for separation by size exclusion was GF-250Xl column 21.2 mm ID X 250 mm (Zorbax).The mobile phase is 0.2 M di-sodium phosphate, pH 7.0. injected sample 200 μl . and detection was by UV absorption at 210 nm. The fraction collector was Foxy Jr (Isco, Inc., USA)

4-6-Optimization of the pH of *M. gallisepticum* culture

Different pH of *M. gallisepticum* culture (7.4, 6.4, 5.7 , 5.45 , 5.25 and 5 pH) were used to obtain the highest peaks of separated protein, the column employed for separation by size exclusion was GF-250Xl column 21.2 mm ID X 250 mm (Zorbax).The mobile phase is 0.2 M sodium phosphate, pH 7.0 . injected sample 200 μl . and The flow rate 5 ml / min detection was by UV absorption at 210 nm. The fraction collector was Foxy Jr (Isco, Inc., USA)

5-Analysis of Eluted Fractions:

A- SDS-PAGE. for the eluted fractions.

Electrophoresis was performed as described by Laemmli (1970). The gels were stained with Co-

massie brilliant blue R-250 (Sigma), and de-stained with mixture of 45% methanol, 10% acetic acid and 45% distilled water .

B-Immunological activity of the eluted fractions.

Electrophoretic transfer to nitrocellulose (NTC) paper was accomplished as described by Towbin et al., (1979). After transfer to NTC, the membranes were stained with Ponceau's (Sigma), and the molecular weight standards were marked. The NTC blots were blocked with 5 % bovine serum albumin (BSA), 20 % fetal bovine serum, in Tris-buffer saline (10 mM Tris, pH 7.3, 0.9% NaCl), for 1 hr at 37°C with gentle rocking, The Mg anti-sera was diluted (1:500) in TBST (10 mM Tris pH 7.2, 150 mM NaCl, 0.05% Tween 20). Secondary antibody (horse radish peroxidase) conjugate was diluted 1:1000. The blots were developed for 5-10 minutes using H₂O₂ and 4-chloro-1-naphthol substrate.

6-Calculations: resolution for two adjacent peaks (m&n) can be calculated as follows:

$$\text{Resolution} = \frac{2(t_n - t_m)}{w_n + w_m}$$

(t_n, t_m retention times w_n, w_m-band width)

RESULTS AND DISCUSSION

The LP 64 kDa is a lipoprotein playing major role in cytheadherence of *Mycoplasma gallisepticum* (Forsyth et. al., 1992), so it is the part of cell

responsible for its virulence. For obtaining this lipoprotein by HPLC the most suitable method was the size exclusion technique in order to obtain this lipoprotein according to its molecular weight (64 kDa).

Optimization of the HPLC in separation and purification of LP 64 kDa band was studied including: wavelength , the mobile phase, sample injection, flow rate and pH of *M. gallisepticum* culture.

Optimization of the HPLC of wavelength on peak height using different wavelengths 210 nm , 220nm , 240 nm and 280 nm., results revealed that 210 nm gave the best peak height 58.91 mAU (Table 1) and the best resolution (Fig. 3). These results were in agreement with Calam and Davidson (1984), Laing et al., (2001) and Hayakawa et al., (2001).

To overcome the electrostatic effects between mobile phase and size-exclusion column matrix to improve the chromatographic profiles two aqueous buffers containing 100 mM NaH₂PO₄ and 200 mM NaH₂PO₄ were used; the aqueous buffer containing 200 mM NaH₂PO₄ improved the chromatographic profiles (Table, 2). The use of buffer ionic strength in range of (0.05-0.5M) was found to be suitable to avoid interaction between solute and matrix, this was in agreement with Jansen and Ryden (1998), also Laing et al, (2001) discovered that albumin or myoglobin had proper

size exclusion behavior at concentrations from 25 to 300 mM.

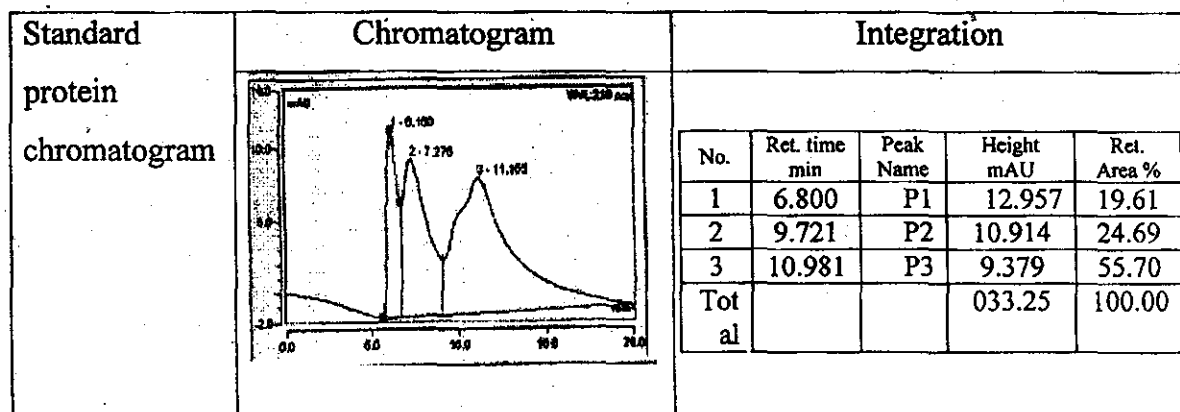
Different injection volumes were tried 20, 40, 200, 300 μ l to optimize the injection volume of protein sample on peak height. The injection of 200 μ l resulted in peak height of 657.21 mAU and increased the resolution of the band (Fig. 4), while in case of injection of 20 μ l, 40 μ l, 300 μ l the peak heights were 28.47, 58.91 and 510 mAU, respectively (Table 3).

The effect of varying flow rates on peak height, protein profile resolution revealed that the decrease of flow rate from 15 ml/min to 5 ml/min, increase peak height from 55.41 mAU to 114.63 mAU and also increase in the resolution of the curve (Table 4) and (Fig. 5).

The effect of pH of *Mycoplasma gallisepticum* culture used for separation revealed that the optimum pH was 5.7 which gave best result of peak height 173.55 mAU and best curve resolution (Table 5) and (Fig. 6).

Electrophoretic pattern and immunoblot of *M. gallisepticum* protein fractions separated by HPLC revealed the presence of LP 64 kDa in fraction 3 with high concentration and pure form (Figures 1 and 2).

In conclusion LP 64 kDa lipoprotein of *Mycoplasma gallisepticum* have been separated by size-exclusion (SEC) HPLC (GF-250X1 column 21.2 mm ID X 250 mm (Zorbax)). Mobile phase was 0.2 M sodium phosphate, pH was 5.7. The flow rate was 5 ml / min. and detection was by UV absorption at 210 nm. and recovered with its immunological activity as seen by immunoblot. The results obtained by SDS-PAGE suggests that this may be a valuable method for isolation and purification as we could separate the target lipoprotein in a pure form, so we can use HPLC (SEC) as a simple, convenient, rapid, reproducible, and reliable method for separation of *M. gallisepticum* LP 64 kDa in highly pure form.



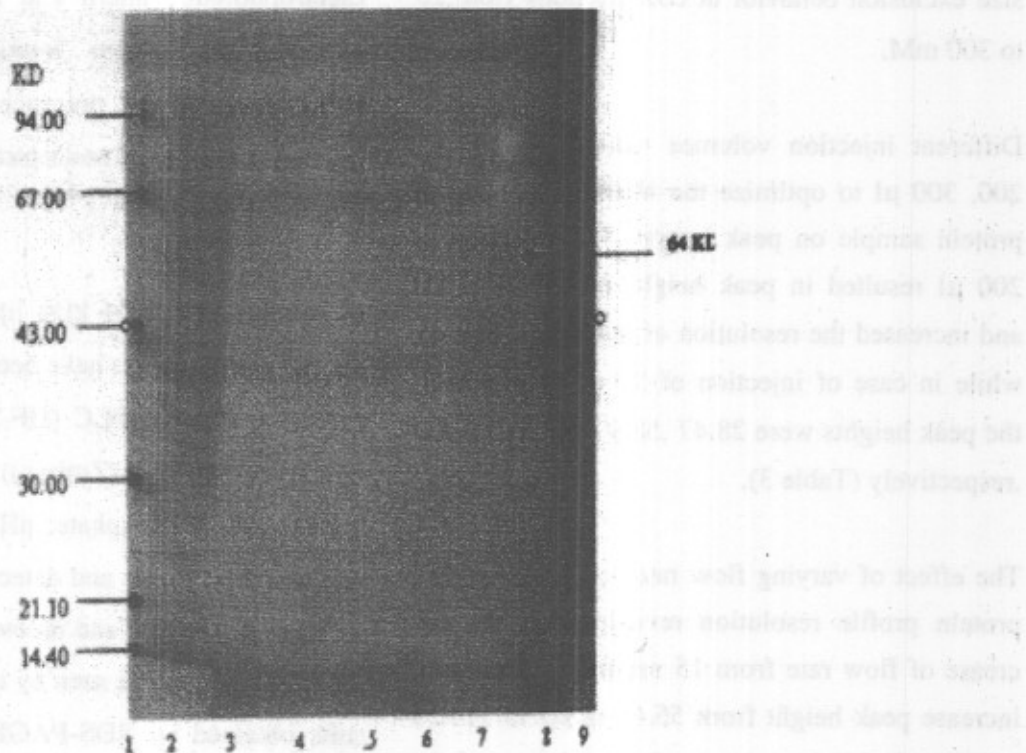


Fig. (1): Electrophoretic pattern of *M. gallisepticum* protein fractions separated by HPLC.

Lane 1: low molecular weight standard (Pharmacia)

Lane 2: *M. gallisepticum* whole cell protein

Lane 3: *M. gallisepticum* (fraction 1, batch 1)

Lane 4: *M. gallisepticum* (fraction 2, batch 1)

Lane 5: *M. gallisepticum* (fraction 3, batch 1)

Lane 6: *M. gallisepticum* (fraction 1, batch 2)

Lane 7: *M. gallisepticum* (fraction 2, batch 2)

Lane 8: *M. gallisepticum* (fraction 3, batch 2)

Lane 9: *M. gallisepticum* whole cell protein.

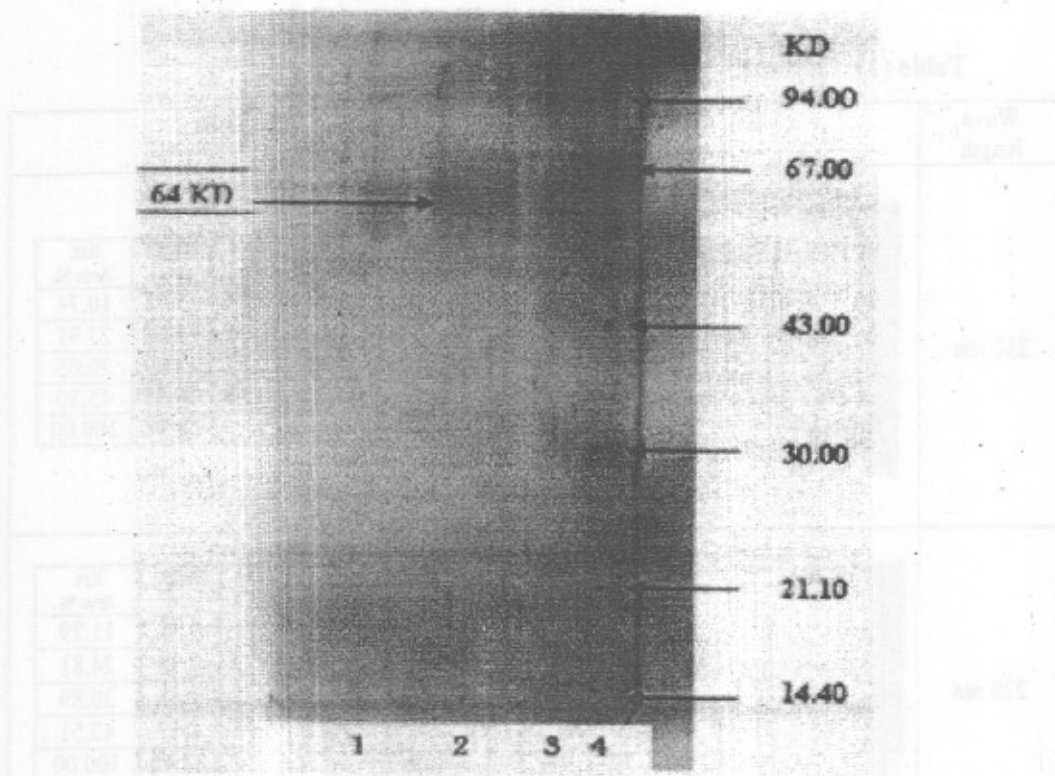


Fig. (2): Immunoblot of *M. gallisepticum* protein fractions separated by HPLC against *M. gallisepticum* positive serum.

Lane 1: *M. gallisepticum* (Fraction 3).

Lane 2: *M. gallisepticum* (Fraction 2).

Lane 3: *M. gallisepticum* (Fraction 1).

Lane 4: Low Molecular weight standard (Pharmacia).

Table (1): Optimization of the wavelength.

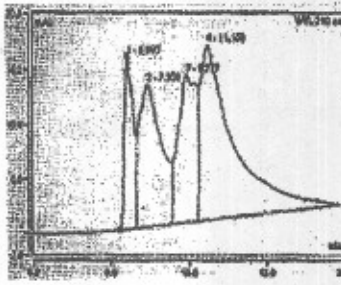
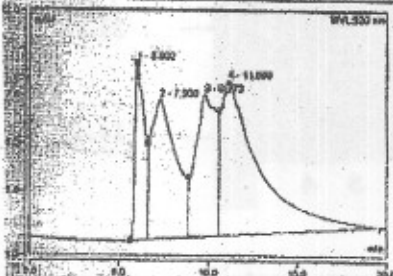
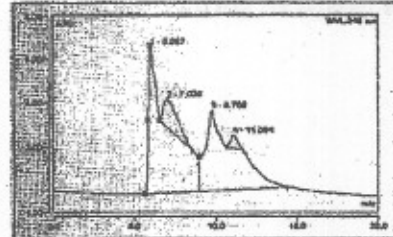
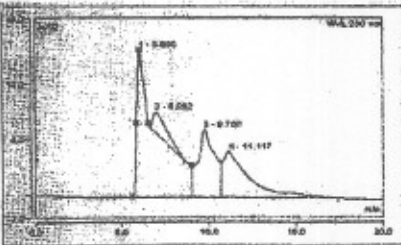
| Wave length | Chromatogram | Integration | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|-------------|---|--|------------|---------------|-----------|------------|-------------|---|------|----|-------|-------|---|------|----|-------|-------|---|------|----|-------|-------|---|-------|----|-------|-------|-------|--|--|-------|--------|
| 210 nm |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. Time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>5.99</td> <td>P1</td> <td>15.93</td> <td>10.74</td> </tr> <tr> <td>2</td> <td>7.31</td> <td>P2</td> <td>13.25</td> <td>23.91</td> </tr> <tr> <td>3</td> <td>9.78</td> <td>P3</td> <td>13.69</td> <td>20.05</td> </tr> <tr> <td>4</td> <td>11.11</td> <td>P4</td> <td>16.05</td> <td>45.30</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>58.91</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | 1 | 5.99 | P1 | 15.93 | 10.74 | 2 | 7.31 | P2 | 13.25 | 23.91 | 3 | 9.78 | P3 | 13.69 | 20.05 | 4 | 11.11 | P4 | 16.05 | 45.30 | Total | | | 58.91 | 100.00 |
| No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 5.99 | P1 | 15.93 | 10.74 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 7.31 | P2 | 13.25 | 23.91 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 9.78 | P3 | 13.69 | 20.05 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 11.11 | P4 | 16.05 | 45.30 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 58.91 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 220 nm |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. Time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>5.99</td> <td>P1</td> <td>9.38</td> <td>11.79</td> </tr> <tr> <td>2</td> <td>7.30</td> <td>P2</td> <td>7.38</td> <td>24.81</td> </tr> <tr> <td>3</td> <td>9.77</td> <td>P3</td> <td>7.53</td> <td>20.89</td> </tr> <tr> <td>4</td> <td>11.09</td> <td>P4</td> <td>8.17</td> <td>42.51</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>32.46</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | 1 | 5.99 | P1 | 9.38 | 11.79 | 2 | 7.30 | P2 | 7.38 | 24.81 | 3 | 9.77 | P3 | 7.53 | 20.89 | 4 | 11.09 | P4 | 8.17 | 42.51 | Total | | | 32.46 | 100.00 |
| No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 5.99 | P1 | 9.38 | 11.79 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 7.30 | P2 | 7.38 | 24.81 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 9.77 | P3 | 7.53 | 20.89 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 11.09 | P4 | 8.17 | 42.51 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 32.46 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 240 nm |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. Time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>5.99</td> <td>P1</td> <td>6.28</td> <td>51.35</td> </tr> <tr> <td>2</td> <td>7.03</td> <td>P2</td> <td>1.25</td> <td>6.77</td> </tr> <tr> <td>3</td> <td>9.77</td> <td>P3</td> <td>3.68</td> <td>40.38</td> </tr> <tr> <td>4</td> <td>11.09</td> <td>P4</td> <td>0.61</td> <td>1.50</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>12.26</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | 1 | 5.99 | P1 | 6.28 | 51.35 | 2 | 7.03 | P2 | 1.25 | 6.77 | 3 | 9.77 | P3 | 3.68 | 40.38 | 4 | 11.09 | P4 | 0.61 | 1.50 | Total | | | 12.26 | 100.00 |
| No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 5.99 | P1 | 6.28 | 51.35 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 7.03 | P2 | 1.25 | 6.77 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 9.77 | P3 | 3.68 | 40.38 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 11.09 | P4 | 0.61 | 1.50 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 12.26 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 280 nm |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. Time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>5.99</td> <td>P1</td> <td>13.31</td> <td>53.82</td> </tr> <tr> <td>2</td> <td>7.39</td> <td>P2</td> <td>1.9</td> <td>5.44</td> </tr> <tr> <td>3</td> <td>9.75</td> <td>P3</td> <td>6.08</td> <td>19.36</td> </tr> <tr> <td>4</td> <td>11.12</td> <td>P4</td> <td>4.10</td> <td>21.38</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>25.39</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | 1 | 5.99 | P1 | 13.31 | 53.82 | 2 | 7.39 | P2 | 1.9 | 5.44 | 3 | 9.75 | P3 | 6.08 | 19.36 | 4 | 11.12 | P4 | 4.10 | 21.38 | Total | | | 25.39 | 100.00 |
| No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 5.99 | P1 | 13.31 | 53.82 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 7.39 | P2 | 1.9 | 5.44 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 9.75 | P3 | 6.08 | 19.36 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 11.12 | P4 | 4.10 | 21.38 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 25.39 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Table (2): Optimization of mobile phase concentration.

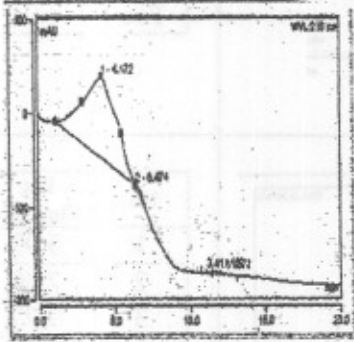
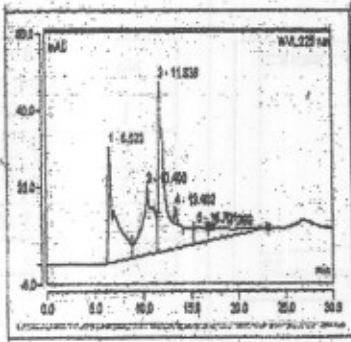
| Mobile phase Conc. | Chromatogram | Integration | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|--|--|--|------------|---------------|-----------|------------|-------------|---|-------|----|--------|-------|---|--------|----|--------|-------|---|--------|----|--------|-------|-------|--------|----|-------|--------|---|--------|----|-------|------|---|--------|----|-------|------|-------|--|--|---------|--------|
| 0.1 mNa H ₂ PO ₄ |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. Time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>6.87</td> <td>P1</td> <td>25.96</td> <td>85.14</td> </tr> <tr> <td>2</td> <td>11.04</td> <td>P2</td> <td>4.56</td> <td>14.44</td> </tr> <tr> <td>3</td> <td>19.72</td> <td>P3</td> <td>0.00</td> <td>0.42</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>30.53</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | 1 | 6.87 | P1 | 25.96 | 85.14 | 2 | 11.04 | P2 | 4.56 | 14.44 | 3 | 19.72 | P3 | 0.00 | 0.42 | Total | | | 30.53 | 100.00 | | | | | | | | | | | | | | | |
| No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 6.87 | P1 | 25.96 | 85.14 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 11.04 | P2 | 4.56 | 14.44 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 19.72 | P3 | 0.00 | 0.42 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 30.53 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 0.2 mNa H ₂ PO ₄ |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. Time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>6.522</td> <td>P1</td> <td>30.062</td> <td>24.12</td> </tr> <tr> <td>2</td> <td>10.490</td> <td>P2</td> <td>17.573</td> <td>22.25</td> </tr> <tr> <td>3</td> <td>11.838</td> <td>P3</td> <td>44.219</td> <td>37.64</td> </tr> <tr> <td>4</td> <td>13.402</td> <td>P4</td> <td>4.128</td> <td>1.12</td> </tr> <tr> <td>5</td> <td>15.761</td> <td>P5</td> <td>4.386</td> <td>5.33</td> </tr> <tr> <td>6</td> <td>17.389</td> <td>P6</td> <td>3.019</td> <td>9.54</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>103.388</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | 1 | 6.522 | P1 | 30.062 | 24.12 | 2 | 10.490 | P2 | 17.573 | 22.25 | 3 | 11.838 | P3 | 44.219 | 37.64 | 4 | 13.402 | P4 | 4.128 | 1.12 | 5 | 15.761 | P5 | 4.386 | 5.33 | 6 | 17.389 | P6 | 3.019 | 9.54 | Total | | | 103.388 | 100.00 |
| No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 6.522 | P1 | 30.062 | 24.12 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 10.490 | P2 | 17.573 | 22.25 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 11.838 | P3 | 44.219 | 37.64 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 13.402 | P4 | 4.128 | 1.12 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 | 15.761 | P5 | 4.386 | 5.33 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 | 17.389 | P6 | 3.019 | 9.54 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 103.388 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Table (1): Optimization of the wavelength.

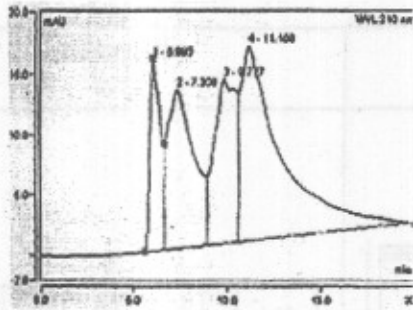
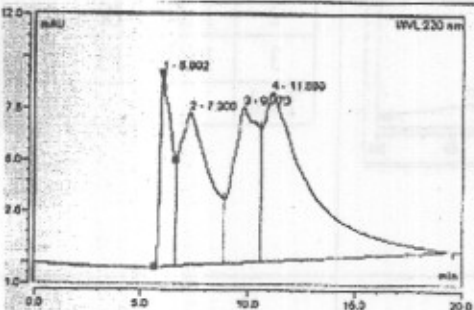
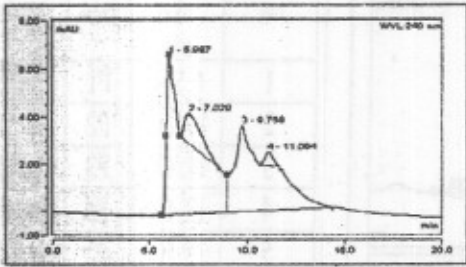
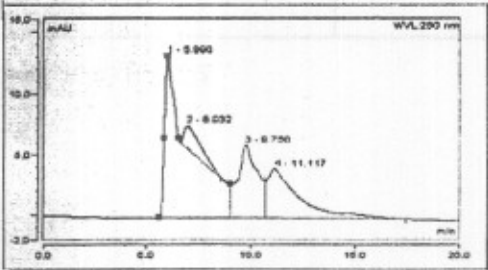
| Wave length | Chromatogram | Integration | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|-------------|---|--|------------|---------------|-----------|------------|-------------|---|------|----|-------|-------|---|------|----|-------|-------|---|------|----|-------|-------|---|-------|----|-------|-------|-------|--|--|-------|--------|
| 210 nm |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. Time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>5.99</td> <td>P1</td> <td>15.93</td> <td>10.74</td> </tr> <tr> <td>2</td> <td>7.31</td> <td>P2</td> <td>13.25</td> <td>23.91</td> </tr> <tr> <td>3</td> <td>9.78</td> <td>P3</td> <td>13.69</td> <td>20.05</td> </tr> <tr> <td>4</td> <td>11.11</td> <td>P4</td> <td>16.05</td> <td>45.30</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>58.91</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | 1 | 5.99 | P1 | 15.93 | 10.74 | 2 | 7.31 | P2 | 13.25 | 23.91 | 3 | 9.78 | P3 | 13.69 | 20.05 | 4 | 11.11 | P4 | 16.05 | 45.30 | Total | | | 58.91 | 100.00 |
| No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 5.99 | P1 | 15.93 | 10.74 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 7.31 | P2 | 13.25 | 23.91 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 9.78 | P3 | 13.69 | 20.05 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 11.11 | P4 | 16.05 | 45.30 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 58.91 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 220 nm |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. Time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>5.99</td> <td>P1</td> <td>9.38</td> <td>11.79</td> </tr> <tr> <td>2</td> <td>7.30</td> <td>P2</td> <td>7.38</td> <td>24.81</td> </tr> <tr> <td>3</td> <td>9.77</td> <td>P3</td> <td>7.53</td> <td>20.89</td> </tr> <tr> <td>4</td> <td>11.09</td> <td>P4</td> <td>8.17</td> <td>42.51</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>32.46</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | 1 | 5.99 | P1 | 9.38 | 11.79 | 2 | 7.30 | P2 | 7.38 | 24.81 | 3 | 9.77 | P3 | 7.53 | 20.89 | 4 | 11.09 | P4 | 8.17 | 42.51 | Total | | | 32.46 | 100.00 |
| No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 5.99 | P1 | 9.38 | 11.79 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 7.30 | P2 | 7.38 | 24.81 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 9.77 | P3 | 7.53 | 20.89 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 11.09 | P4 | 8.17 | 42.51 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 32.46 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 240 nm |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. Time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>5.99</td> <td>P1</td> <td>6.28</td> <td>51.35</td> </tr> <tr> <td>2</td> <td>7.03</td> <td>P2</td> <td>1.25</td> <td>6.77</td> </tr> <tr> <td>3</td> <td>9.77</td> <td>P3</td> <td>3.68</td> <td>40.38</td> </tr> <tr> <td>4</td> <td>11.09</td> <td>P4</td> <td>0.61</td> <td>1.50</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>12.26</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | 1 | 5.99 | P1 | 6.28 | 51.35 | 2 | 7.03 | P2 | 1.25 | 6.77 | 3 | 9.77 | P3 | 3.68 | 40.38 | 4 | 11.09 | P4 | 0.61 | 1.50 | Total | | | 12.26 | 100.00 |
| No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 5.99 | P1 | 6.28 | 51.35 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 7.03 | P2 | 1.25 | 6.77 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 9.77 | P3 | 3.68 | 40.38 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 11.09 | P4 | 0.61 | 1.50 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 12.26 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 280 nm |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. Time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>5.99</td> <td>P1</td> <td>13.31</td> <td>53.82</td> </tr> <tr> <td>2</td> <td>7.39</td> <td>P2</td> <td>1.9</td> <td>5.44</td> </tr> <tr> <td>3</td> <td>9.75</td> <td>P3</td> <td>6.08</td> <td>19.36</td> </tr> <tr> <td>4</td> <td>11.12</td> <td>P4</td> <td>4.10</td> <td>21.38</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>25.39</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | 1 | 5.99 | P1 | 13.31 | 53.82 | 2 | 7.39 | P2 | 1.9 | 5.44 | 3 | 9.75 | P3 | 6.08 | 19.36 | 4 | 11.12 | P4 | 4.10 | 21.38 | Total | | | 25.39 | 100.00 |
| No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 5.99 | P1 | 13.31 | 53.82 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 7.39 | P2 | 1.9 | 5.44 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 9.75 | P3 | 6.08 | 19.36 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 11.12 | P4 | 4.10 | 21.38 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 25.39 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Table (2): Optimization of the mobile phase concentration.

Table (4): Optimization of flow rate.

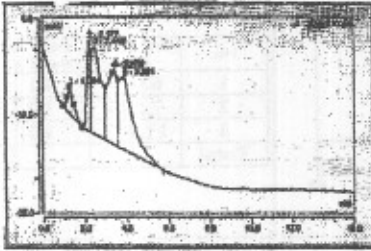
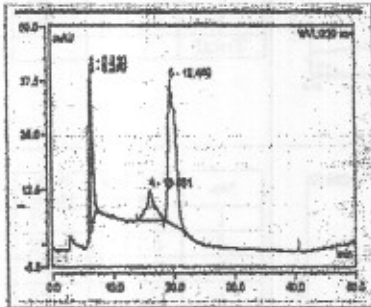
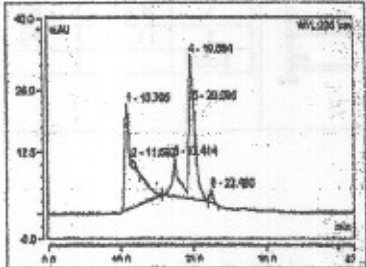
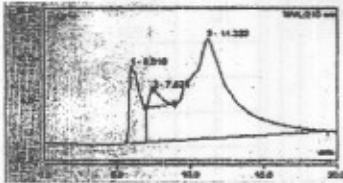
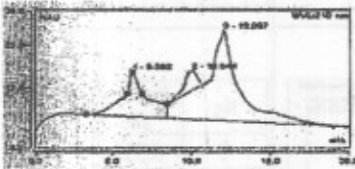
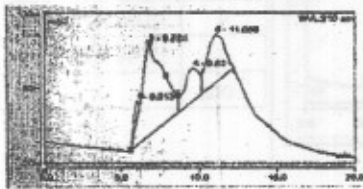
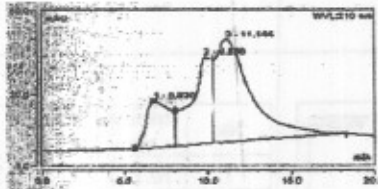
| flow Rate | Chromatogram | Integration | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|-----------------|---|--|------------|---------------|-----------|------------|-------------|---|--------|----|--------|-------|---|--------|----|-------|-------|---|--------|----|-------|-------|---|--------|----|--------|-------|---|--------|----|-------|-------|-------|--|--|--------|--------|
| Flow 15 ml/ min |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>1.26</td> <td>P1</td> <td>4.11</td> <td>5.37</td> </tr> <tr> <td>2</td> <td>2.18</td> <td>P2</td> <td>14.05</td> <td>10.05</td> </tr> <tr> <td>3</td> <td>2.44</td> <td>P3</td> <td>13.58</td> <td>27.96</td> </tr> <tr> <td>4</td> <td>3.40</td> <td>P4</td> <td>11.75</td> <td>22.82</td> </tr> <tr> <td>5</td> <td>3.86</td> <td>P5</td> <td>11.24</td> <td>33.51</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>55.41</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. time min | Peak Name | Height mAU | Ret. Area % | 1 | 1.26 | P1 | 4.11 | 5.37 | 2 | 2.18 | P2 | 14.05 | 10.05 | 3 | 2.44 | P3 | 13.58 | 27.96 | 4 | 3.40 | P4 | 11.75 | 22.82 | 5 | 3.86 | P5 | 11.24 | 33.51 | Total | | | 55.41 | 100.00 |
| No. | Ret. time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 1.26 | P1 | 4.11 | 5.37 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 2.18 | P2 | 14.05 | 10.05 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 2.44 | P3 | 13.58 | 27.96 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 3.40 | P4 | 11.75 | 22.82 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 | 3.86 | P5 | 11.24 | 33.51 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 55.41 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Flow 5 ml/ min |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>6.310</td> <td>P1</td> <td>36.81</td> <td>13.60</td> </tr> <tr> <td>2</td> <td>6.342</td> <td>P2</td> <td>36.65</td> <td>16.73</td> </tr> <tr> <td>3</td> <td>6.370</td> <td>P3</td> <td>0.843</td> <td>0.14</td> </tr> <tr> <td>4</td> <td>16.061</td> <td>P4</td> <td>6.77</td> <td>13.61</td> </tr> <tr> <td>5</td> <td>19.449</td> <td>P5</td> <td>33.18</td> <td>55.91</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>114.63</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. time min | Peak Name | Height mAU | Ret. Area % | 1 | 6.310 | P1 | 36.81 | 13.60 | 2 | 6.342 | P2 | 36.65 | 16.73 | 3 | 6.370 | P3 | 0.843 | 0.14 | 4 | 16.061 | P4 | 6.77 | 13.61 | 5 | 19.449 | P5 | 33.18 | 55.91 | Total | | | 114.63 | 100.00 |
| No. | Ret. time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 6.310 | P1 | 36.81 | 13.60 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 6.342 | P2 | 36.65 | 16.73 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 6.370 | P3 | 0.843 | 0.14 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 16.061 | P4 | 6.77 | 13.61 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 | 19.449 | P5 | 33.18 | 55.91 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 114.63 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Flow 3 ml/ min |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>10.795</td> <td>P1</td> <td>21.476</td> <td>46.07</td> </tr> <tr> <td>2</td> <td>11.592</td> <td>P2</td> <td>1.320</td> <td>1.52</td> </tr> <tr> <td>3</td> <td>17.414</td> <td>P3</td> <td>7.830</td> <td>12.70</td> </tr> <tr> <td>4</td> <td>19.684</td> <td>P4</td> <td>29.135</td> <td>37.76</td> </tr> <tr> <td>5</td> <td>22.480</td> <td>P5</td> <td>2.539</td> <td>1.95</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>62.300</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. time min | Peak Name | Height mAU | Ret. Area % | 1 | 10.795 | P1 | 21.476 | 46.07 | 2 | 11.592 | P2 | 1.320 | 1.52 | 3 | 17.414 | P3 | 7.830 | 12.70 | 4 | 19.684 | P4 | 29.135 | 37.76 | 5 | 22.480 | P5 | 2.539 | 1.95 | Total | | | 62.300 | 100.00 |
| No. | Ret. time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 10.795 | P1 | 21.476 | 46.07 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 11.592 | P2 | 1.320 | 1.52 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 17.414 | P3 | 7.830 | 12.70 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 19.684 | P4 | 29.135 | 37.76 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 | 22.480 | P5 | 2.539 | 1.95 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 62.300 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Table (5): Optimization of pH of the *M. gallisepticum* culture.

| pH | Chromatogram | Integration | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|-------|---|---|------------|---------------|-----------|------------|-------------|---|-------|----|--------|-------|---|-------|----|--------|-------|---|--------|----|--------|-------|-------|--------|----|--------|--------|-------|--|--|---------|--------|
| 7.4 |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>6.010</td> <td>P1</td> <td>9.145</td> <td>13.48</td> </tr> <tr> <td>2</td> <td>7.524</td> <td>P2</td> <td>2.041</td> <td>3.02</td> </tr> <tr> <td>3</td> <td>11.233</td> <td>P3</td> <td>11.953</td> <td>83.50</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>23.139</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. time min | Peak Name | Height mAU | Ret. Area % | 1 | 6.010 | P1 | 9.145 | 13.48 | 2 | 7.524 | P2 | 2.041 | 3.02 | 3 | 11.233 | P3 | 11.953 | 83.50 | Total | | | 23.139 | 100.00 | | | | | |
| No. | Ret. time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 6.010 | P1 | 9.145 | 13.48 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 7.524 | P2 | 2.041 | 3.02 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 11.233 | P3 | 11.953 | 83.50 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 23.139 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
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| No. | Ret. time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 6.010 | P1 | 12.895 | 23.62 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 7.524 | P2 | 5.630 | 5.90 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 11.233 | P3 | 26.286 | 70.48 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 44.812 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5.7 |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>6.013</td> <td>P1</td> <td>12.500</td> <td>2.15</td> </tr> <tr> <td>2</td> <td>6.791</td> <td>P2</td> <td>85.657</td> <td>58.59</td> </tr> <tr> <td>3</td> <td>9.631</td> <td>P4</td> <td>29.531</td> <td>14.01</td> </tr> <tr> <td>4</td> <td>11.058</td> <td>P5</td> <td>44.795</td> <td>25.25</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>172.483</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. time min | Peak Name | Height mAU | Ret. Area % | 1 | 6.013 | P1 | 12.500 | 2.15 | 2 | 6.791 | P2 | 85.657 | 58.59 | 3 | 9.631 | P4 | 29.531 | 14.01 | 4 | 11.058 | P5 | 44.795 | 25.25 | Total | | | 172.483 | 100.00 |
| No. | Ret. time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 6.013 | P1 | 12.500 | 2.15 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 6.791 | P2 | 85.657 | 58.59 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 9.631 | P4 | 29.531 | 14.01 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 11.058 | P5 | 44.795 | 25.25 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 172.483 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5.45 |  | <table border="1"> <thead> <tr> <th>No.</th> <th>Ret. Time min</th> <th>Peak Name</th> <th>Height mAU</th> <th>Ret. Area %</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>6.820</td> <td>P1</td> <td>16.483</td> <td>16.13</td> </tr> <tr> <td>2</td> <td>9.850</td> <td>P2</td> <td>30.801</td> <td>27.99</td> </tr> <tr> <td>3</td> <td>11.144</td> <td>P3</td> <td>36.636</td> <td>55.88</td> </tr> <tr> <td>Total</td> <td></td> <td></td> <td>83.920</td> <td>100.00</td> </tr> </tbody> </table> | No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | 1 | 6.820 | P1 | 16.483 | 16.13 | 2 | 9.850 | P2 | 30.801 | 27.99 | 3 | 11.144 | P3 | 36.636 | 55.88 | Total | | | 83.920 | 100.00 | | | | | |
| No. | Ret. Time min | Peak Name | Height mAU | Ret. Area % | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 6.820 | P1 | 16.483 | 16.13 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 9.850 | P2 | 30.801 | 27.99 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 11.144 | P3 | 36.636 | 55.88 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Total | | | 83.920 | 100.00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

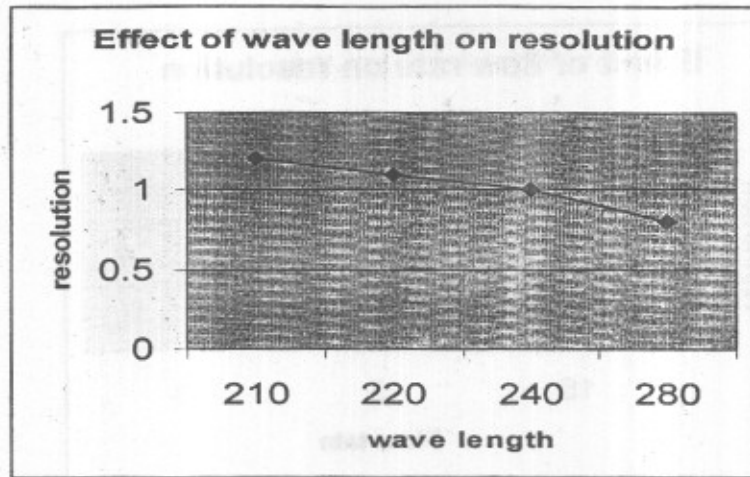


Fig. (3): Effect of wave length on the resolution.

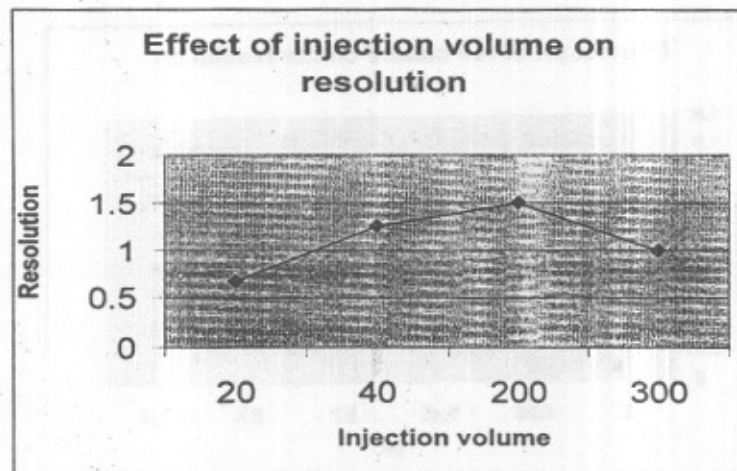


Fig. (4): Effect of injection volume on the resolution.

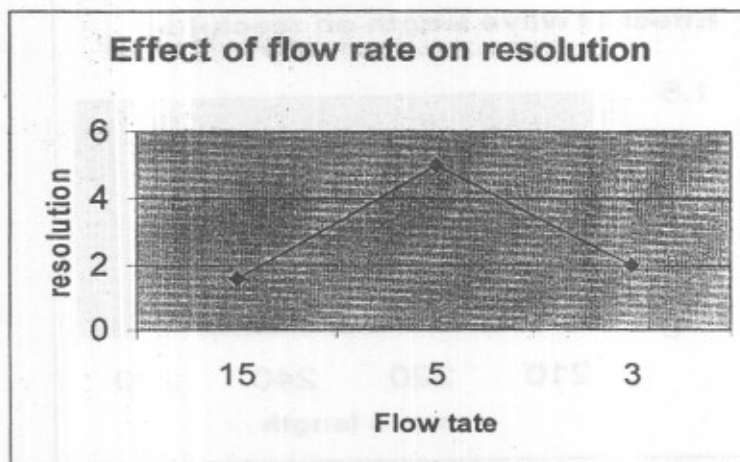


Fig. (5): Effect of flow rate on the resolution.

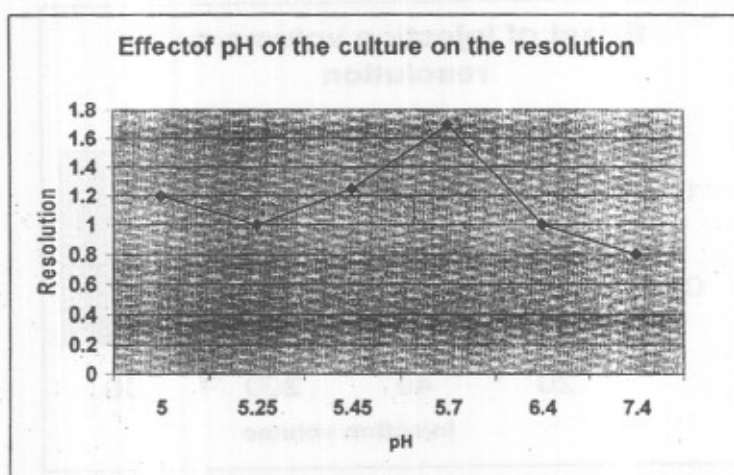


Fig. (6): Effect of pH of the culture on the resolution.

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