CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 DENSITY ANALYSES

Density measurement results of Ekon D®, LaboClean Ft®. and Contrad concentrate® are reported in Table 4.1.

Table 4.1 Density results of Ekon D®, LaboClean Ft®. and Contrad concentrate®

Parameters	Ekon D®	Contrad®	LaboClean Ft®
Temperature (°C)	20	20	20
Density	1.010	1.067	1.302
SG	1.012	1.069	1.305

Temperature (°C), true density (g/ml) and specific gravity of the detergents are reported in Table 4.1. All three parameters were simultaneously determined using the Anton Paar DMA 38 density meter, as described in paragraph 3.3.2.

Specific gravity is a ratio, expressed decimally, of the weight of a substance to the weight of an equal volume of a substance chosen as a standard with both substances at the same temperature or the temperature of each being known (Ansel, 2010). Water is used as the standard for the specific gravities of liquids.

One can multiply milliliters by specific gravity and have grams, because the volume of the liquid in question is assumed to be the same volume as water for which milliliters equals grams (Ansel, 2010). Hence specific gravity results will be used in the quantification of the detergent's used as reference in HPLC analysis for glassware cleaning validation.

Equation by Ansel, (2010):

Grams (other liquid) = Grams (of equal volume of water) x Specific gravity (other liquid)

4.2 SPECTROPHOTOMETRIC ANALYSES

Spectrophotometric analyses were conducted to determine the relative maximum wavelength absorption of Ekon D®, Contrad® and LaboClean Ft. concentrate®. Figure 4.1, 4.2 and 4.3 represent the spectrum graph report of Ekon D®, LaboClean Ft. ® and Contrad concentrate® respectively.

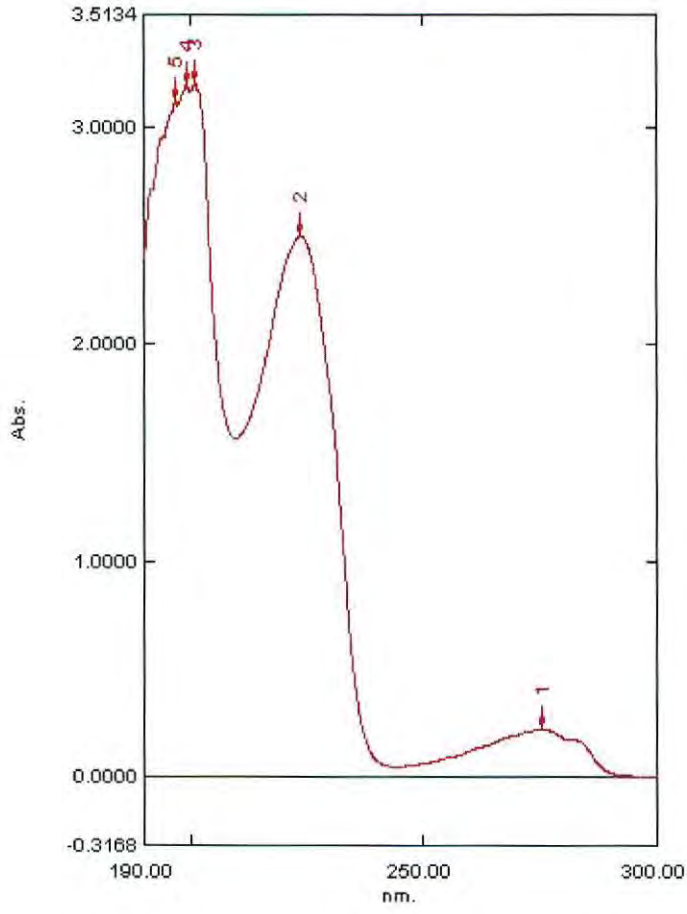

Figure 4.1 UV spectrum obtained for Ekon D concentrate® at concentration 2 mg/ml.

Figure 4.1 is a representative Ekon D concentrate® spectrum graph report at concentration 2 mg/ml. The spectrum graph shows relative high absorbance of the analyte at wavelength range of 215 nm to 225 nm and maximum absorbance at wavelengths ranging from 201 nm to 198 nm.

Figure 4.2 is a representative LaboClean Ft. concentrate® spectrum graph report at concentration 26 mg/ml. The spectrum graph shows relative high wavelength absorption of the analyte at around 290 nm and maximum wavelength absorption at wavelengths ranging from 200 nm to 220 nm.

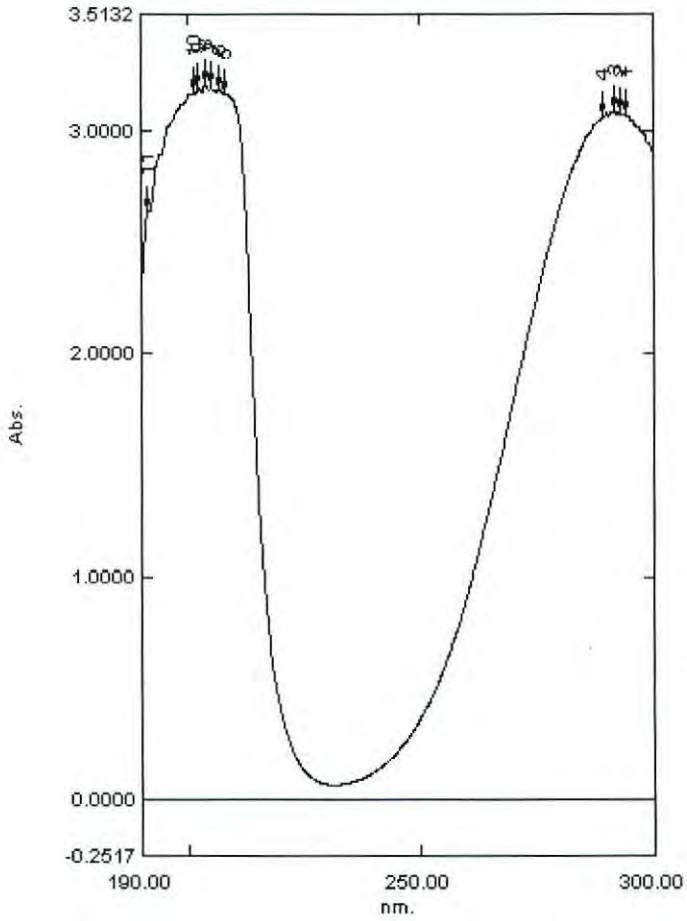

Figure 4.2 UV spectrum obtained for LaboClean Ft. concentrate® at concentration 26 mg/ml.

Figure 4.3 is a representative Contrad concentrate® spectrum graph report at concentration 2 mg/ml. The spectrum graph shows relative high absorbance of the analyte at wavelength of about 220 nm and maximum absorbance at wavelength 200 nm.

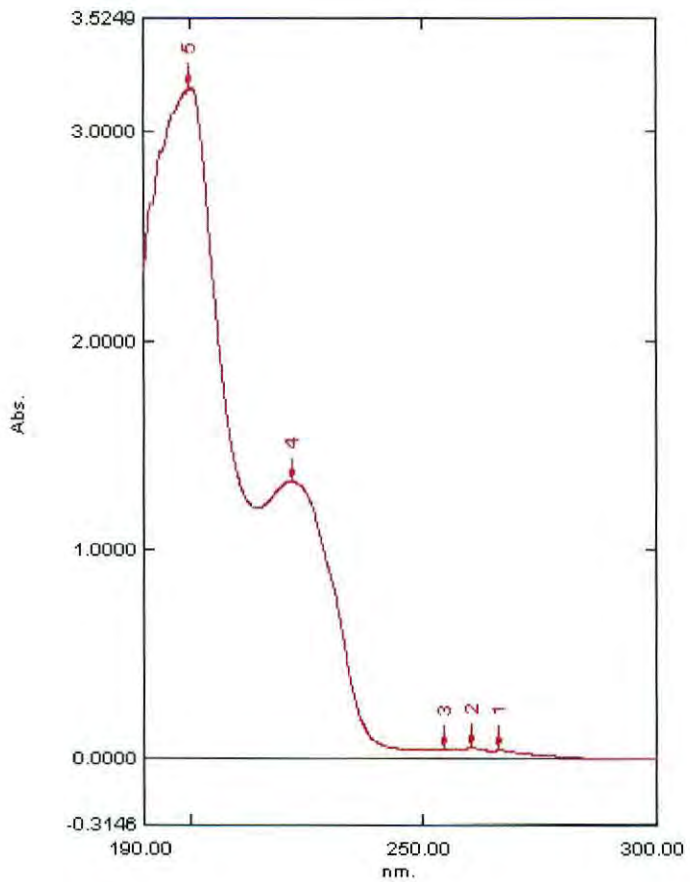

Figure 4.3 UV spectrum obtained for Contrad concentrate® at concentration 2 mg/ml.

4.2.1 Discussion and conclusion of spectrophotometric analyses

Spectrophotometric analyses were conducted to supplement the HPLC method development process. The determination of the relative maximum wavelength absorption of the three detergents served as a guide to choosing a suitable wavelength to be used in HPLC analyses that can accommodate the three detergents while eliminating possible interferences at low wavelengths.

Poor chromatographic response by many of today's molecules above 220 nm-240 nm means that cleaning assays are forced into the lower end of the UV range (Plasz, 2005). Indeed when observing figures 4.1 to 4.3, the three detergent's showed maximum absorption at wavelength around 200 nm. This observation presented an

additional challenge as this response could have resulted from any residual present at the time of observation regardless of whether it was assumed to be the analyte or not. The spectra reports depicted in figures 4.1 to 4.3 also show that ingredients in cleaning products contain weak UV chromophores. One feature was common in the three spectra reports, a rise in the graph at wavelength ranging between 225 nm to 200 nm. This observation led to the decision to choose wavelengths 205 nm and 220 nm as a starting point in HPLC method development analyses.

4.3 HPLC METHOD DEVELOPMENT ANALYSES

This section reports the findings of the experimental procedures followed for the HPLC method development as guided by the literature review discussed in chapter 2.

4.3.1 Analyte

Ekon D®, Contrad® and LaboClean Ft. concentrate® are the three detergents used in the laboratory of study for which detection by the HPLC method developed was key. As mentioned in the literature review in chapter 2, the active chemical composition of the detergents remains an unknown factor in the development of this HPLC method. This matter presented a challenge in the identification of the active ingredient/s of the detergents. Craft *et.al* (2010) says, with non-specific analytical techniques, any residual measured must be assumed to be the active ingredient for cleaning validation. When using specific techniques like HPLC-UV time is a limiting factor to production and does not allow identification and quantification of every peak present.
Solutions with a concentration of about 5% v/v were prepared for each detergent. The solutions were used as standards for the HPLC method development.

4.3.2 Chromatographic mode and column choice

As discussed in paragraph 2.4.3, reversed phase chromatographic mode was chosen as the suitable chromatographic mode for the HPLC method to be developed. As a starting point a µBondapak C_{18} (10 µm) 300 x 3.9 mm column was used as reviewed for its advantages in 2.4.4.

4.3.3 Mobile phase selection

Isocratic analyses were employed when developing the HPLC method. A couple of mobile phase combinations were trialled to determine acceptable chromatographic conditions and chromatography. Figures 4.4 to 4.15 are chromatographic representations of mobile phase combinations trialled and tested for acceptable chromatography.

Mobile phase 1

The first mobile phase was a combination of methanol and Milli-Q water at a ratio of 30:70, the pH of the mobile phase was not adjusted. Figures 4.4 to 4.6 are chromatograms of the detergents obtained with an injection volume of 25 µl at ambient column temperatures at a mobile phase flow rate of 1.0 ml/min. Wavelengths 205 nm and 220 nm were used to evaluate the wavelength that offers an acceptable response of unknown analyte peaks to be identified.

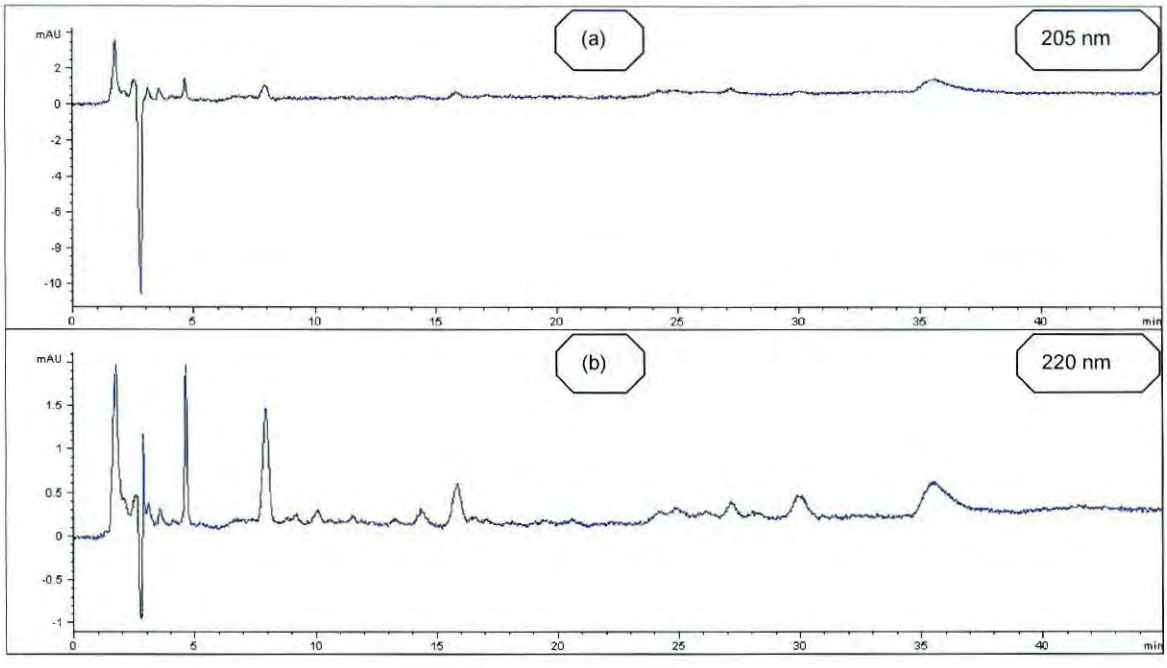

Figure 4.4 Chromatogram obtained with Ekon D concentrate® at a concentration of 5% v/v using mobile phase 1, with chromatograms (a) and (b) obtained at wavelengths 205 nm and 220 nm respectively.

Figure 4.4 is a chromatographic representation of Ekon D concentrate® at 5% v/v concentration. The first chromatogram labelled (a) in figure 4.4 was obtained at wavelength 205 nm and the second chromatogram labelled (b) was obtained at wavelength 220 nm. Chromatogram (b) obtained at 220 nm of figure 4.4 showed a better UV response for the analyte when compared with chromatogram (a) obtained at wavelength 205 nm with the same analyte at the same concentration and chromatographic conditions.

Figure 4.5 is a chromatographic representation of LaboClean FT concentrate® at 5% v/v concentration. Chromatogram (a) in figure 4.5 was obtained at wavelength 205 nm and chromatogram (b) was obtained at wavelength 220 nm. Chromatogram (a) of figure 4.5 obtained at wavelength 205 nm showed a better UV response for the analyte when compared to chromatogram (b) obtained at wavelength 220 nm with the same analyte at the same concentration and chromatographic conditions.

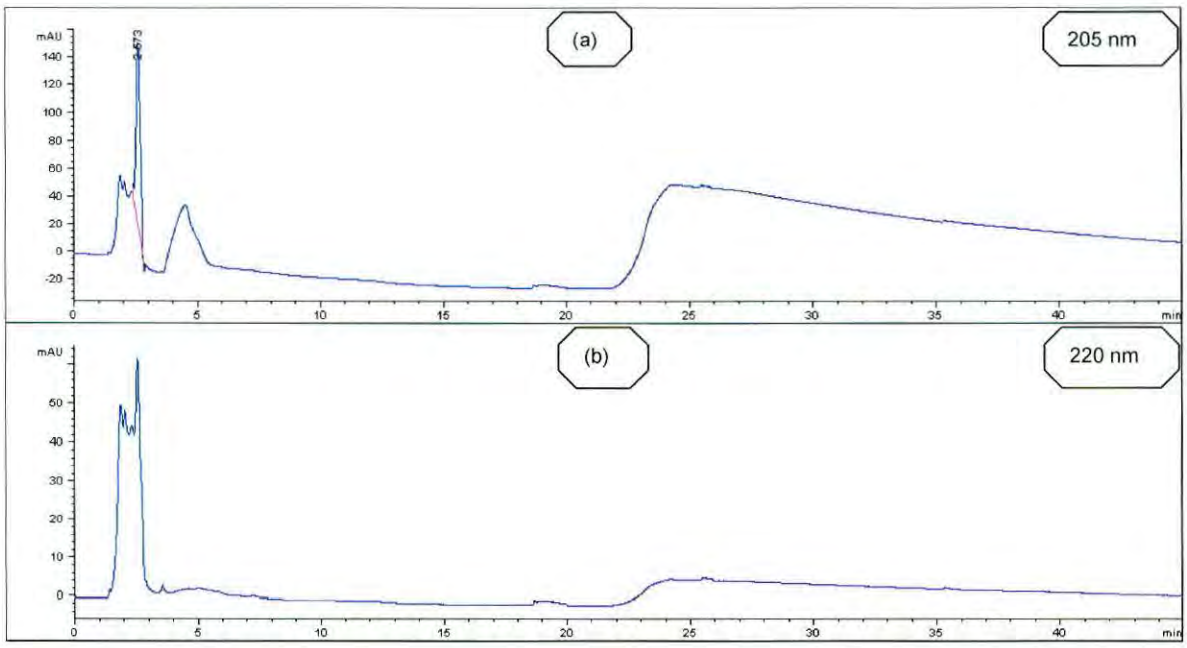

Figure 4.5 Chromatogram obtained with LaboClean FT. concentrate® at a concentration of 5% v/v using mobile phase 1, with chromatograms (a) and (b) obtained at wavelengths 205 nm and 220 nm respectively.

Figure 4.6 is a chromatographic representation of Contrad concentrate® at 5% v/v concentration. Chromatogram (a) of figure 4.6 was obtained at wavelength 205 nm and the chromatogram (b) was obtained at wavelength 220 nm. Chromatogram (a) obtained at wavelength 205 nm of figure 4.6 showed a better UV response for the analyte when compared to chromatogram (b) obtained at wavelength 220 nm with the same analyte at the same concentration and chromatographic conditions.

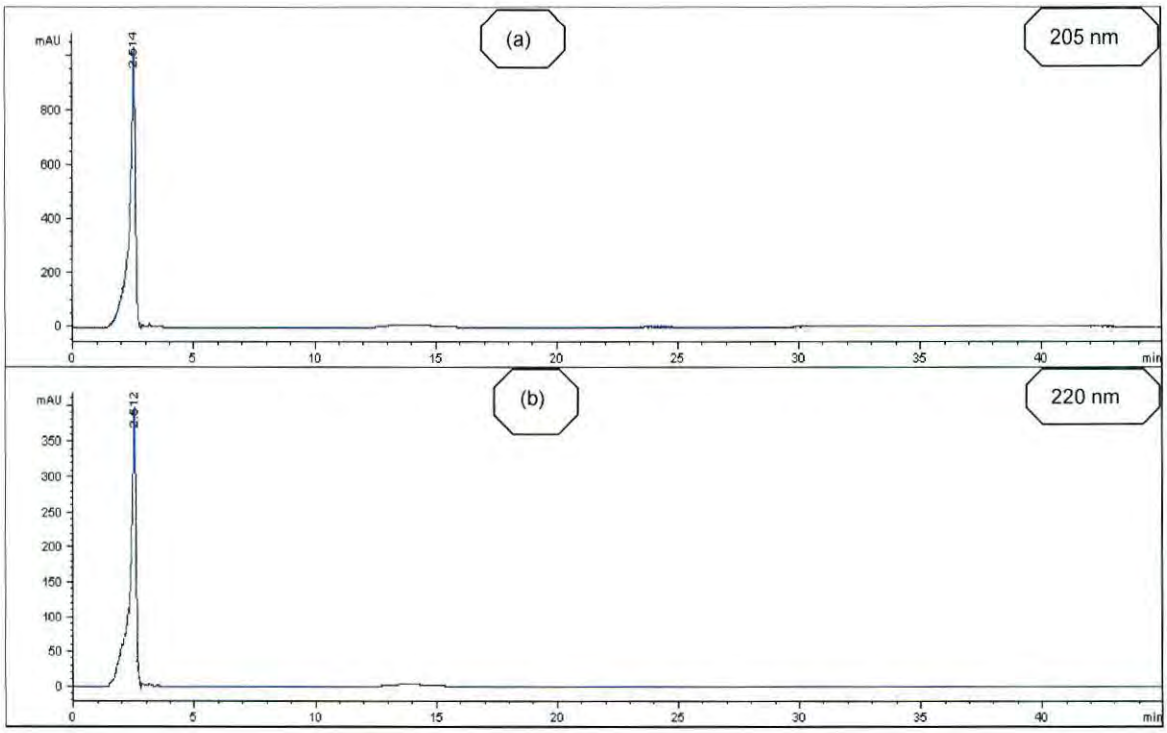

Figure 4.6 Chromatogram obtained with Contrad concentrate® at a concentration of 5% v/v using mobile phase 1, with chromatograms (a) and (b) obtained at wavelengths 205 nm and 220 nm respectively.

Evaluating figures 4.4 to 4.6, the chromatograms were not acceptable for quantification of any of the detergents due to poor chromatographic response to the mobile phase in trial. However, the results confirm the spectrophotometer analyses which showed that the three detergents had a better UV response at low wavelengths.

Mobile phase 2

The second mobile phase tested was a combination of acetonitrile and Milli-Q water at a ratio of 70:30, the pH of the mobile phase was not adjusted. Chromatographic conditions used to trial mobile phase 2 were the same as those employed in mobile phase 1, with an injection volume of 25 µl, using a µBondapak C_{18} (10 µm) 300 x 3.9 mm column at ambient temperature and wavelength set at 205 nm and 220 nm.

Figures 4.7, 4.8 and 4.9 are chromatographic representation of Ekon D®, LaboClean FT® and Contrad® concentrate all at 5% v/v concentration obtained with mobile phase 2 respectively.

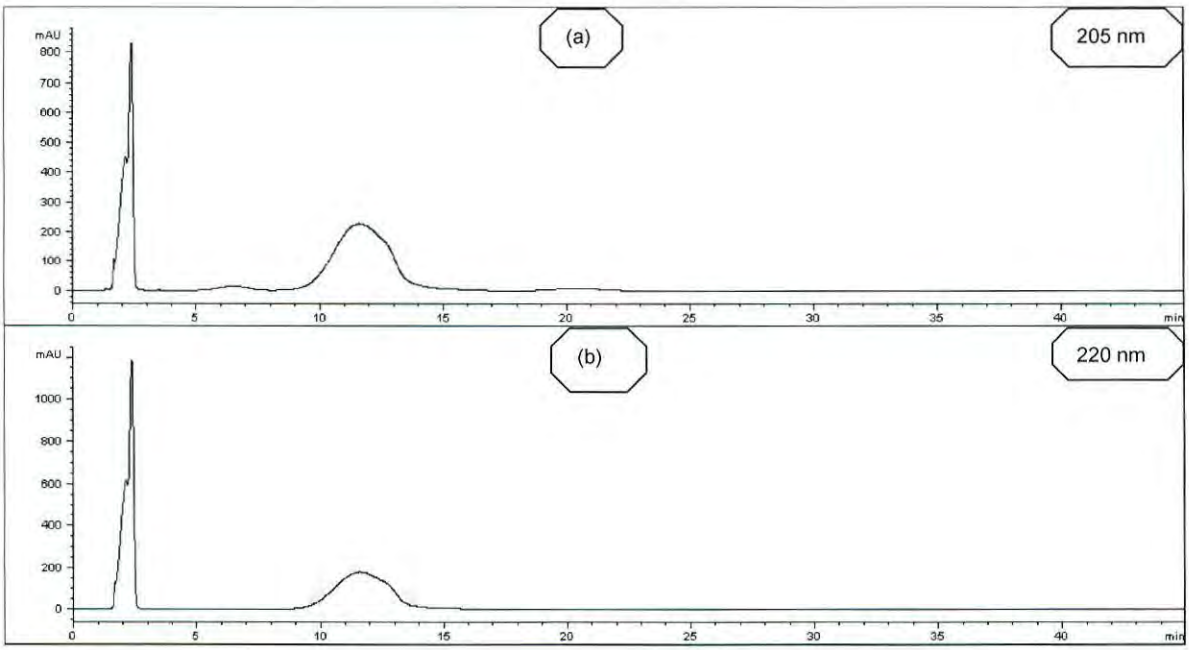

Figure 4.7 Chromatogram obtained with Ekon D concentrate® at a concentration of 5% v/v using mobile phase 2, with chromatograms (a) and (b) obtained at wavelengths 205 nm and 220 nm respectively.

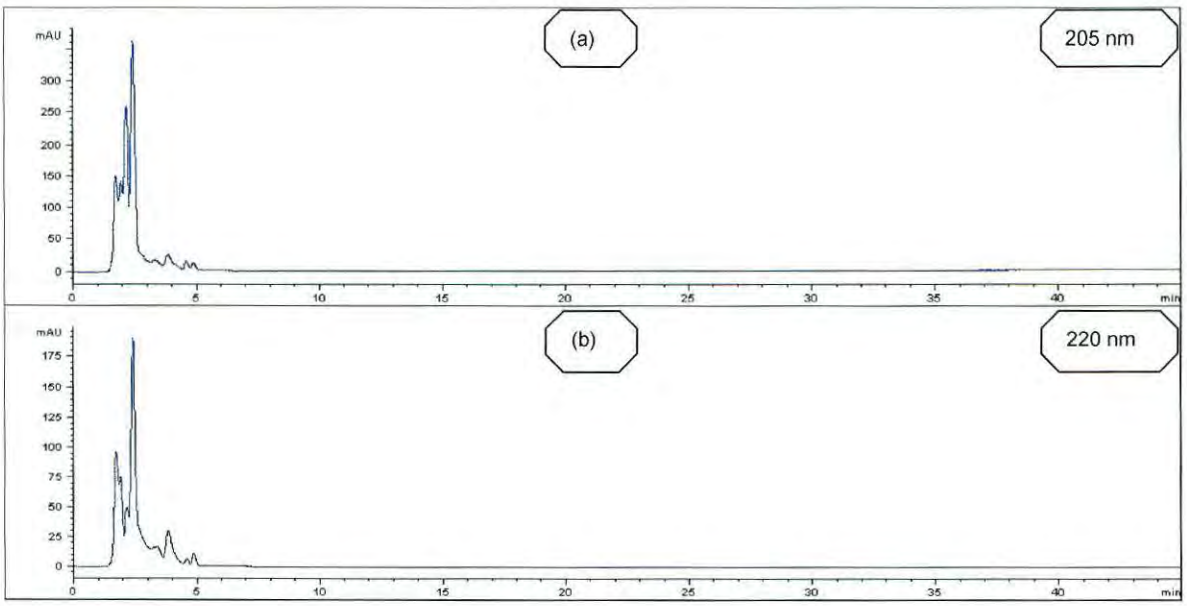

Figure 4.8 Chromatogram obtained with LaboClean FT. concentrate® at a concentration of 5% v/v using mobile phase 2, with chromatograms (a) and (b) obtained at wavelengths 205 nm and 220 nm respectively.

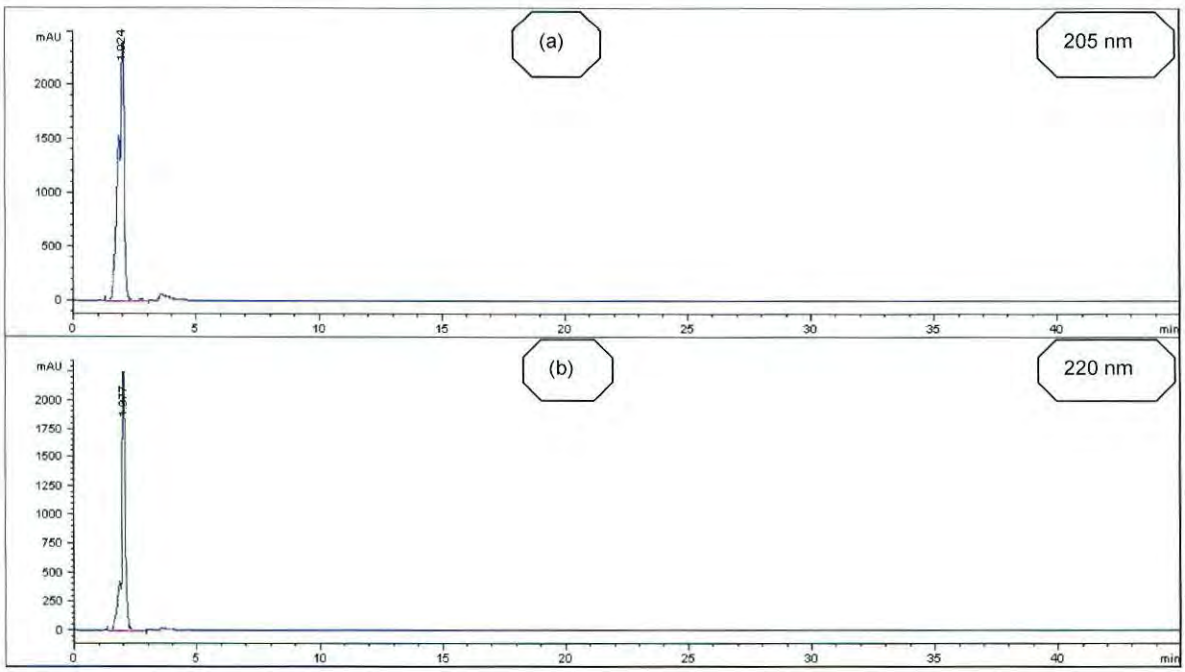

Figure 4.9 Chromatogram obtained with Contrad concentrate® at a concentration of 5% v/v using mobile phase 2, with chromatograms (a) and (b) obtained at wavelengths 205 nm and 220 nm respectively.

Observation of figures 4.7, 4.8 and 4.9, show that Ekon D concentrate® has a better UV response at 220 nm when compared to Contrad® and LaboClean FT concentrate® which showed a better UV response at wavelength 205 nm. An attempt to use the second mobile phase did not resolve the problems encountered with the first mobile phase. Quantification of the active unknown peaks of any of the detergents was not possible.

Mobile phase 3

The third mobile phase used was a combination of methanol and Milli-Q water at a ratio of 30:70, with the pH of the mobile phase adjusted to 3.0 with 85% phosphoric acid. Chromatographic conditions used to trial mobile phase 3 were the same as those employed in mobile phase 1 & 2, with an injection volume of 25 µl, using a µBondapak C_{18} (10 µm) 300 x 3.9 mm column at ambient temperatures and wavelength set at 205 nm and 220 nm. Figures 4.10, 4.11 and 4.12 are chromatographic representation of Ekon D®, LaboClean FT® and Contrad® concentrate all at 5% v/v concentration obtained with mobile phase 3.

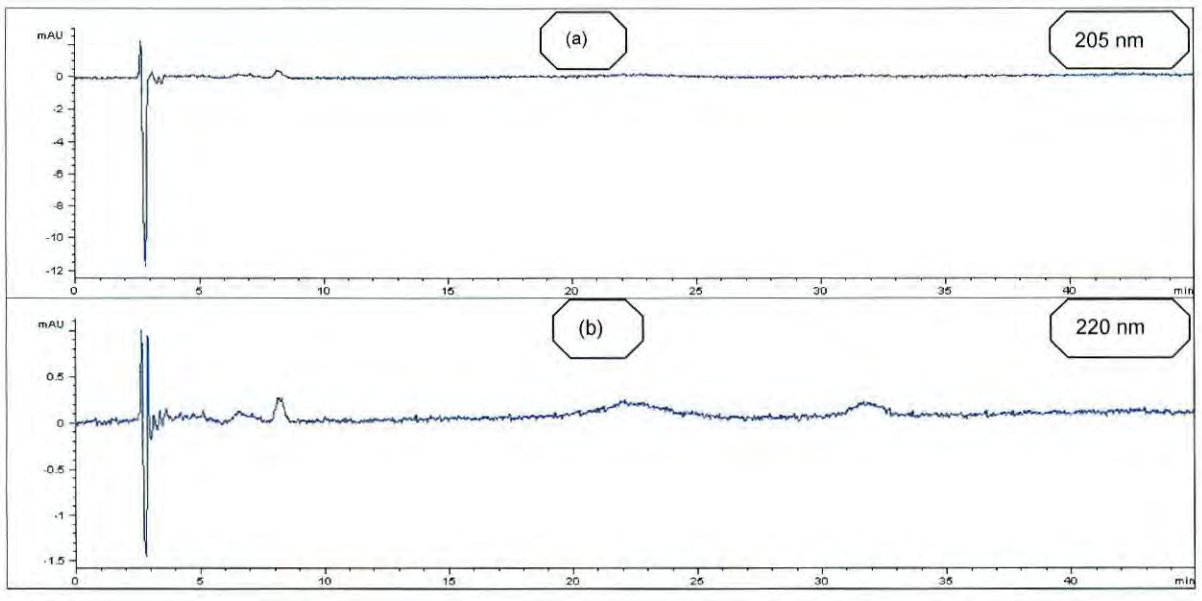

Figure 4.10 Chromatogram obtained with Ekon D concentrate® at a concentration of 5% v/v using mobile phase 3, with chromatograms (a) and (b) obtained at wavelengths 205 nm and 220 nm respectively.

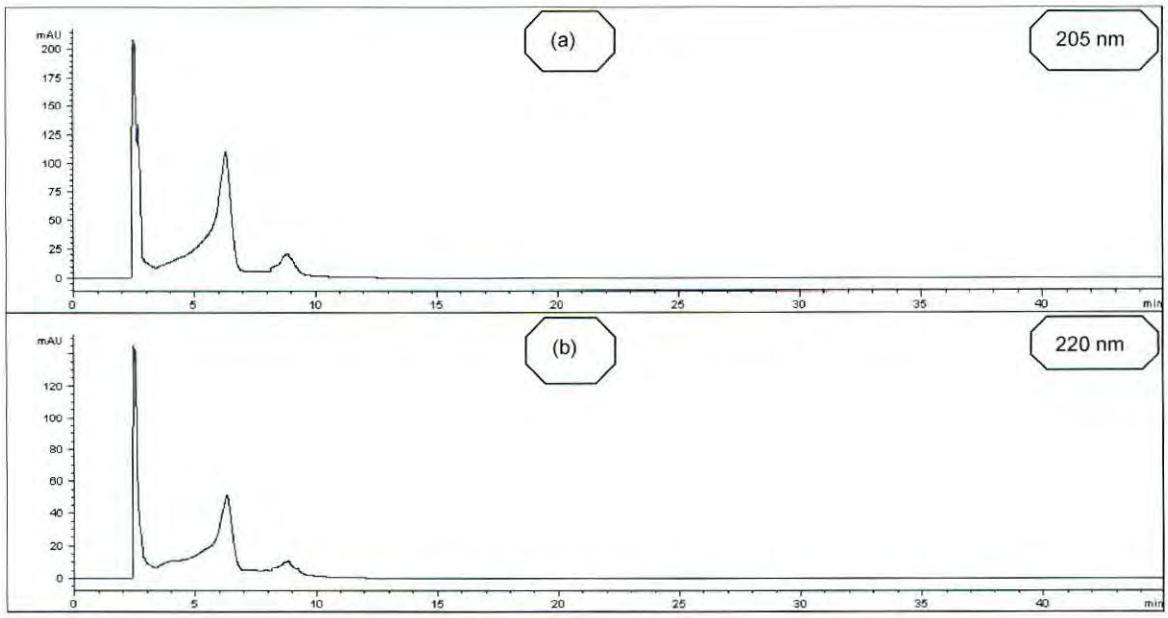

Figure 4.11 Chromatogram obtained with LaboClean FT. concentrate® at a concentration of 5% v/v using mobile phase 3, with chromatograms (a) and (b) obtained at wavelengths 205 nm and 220 nm respectively.

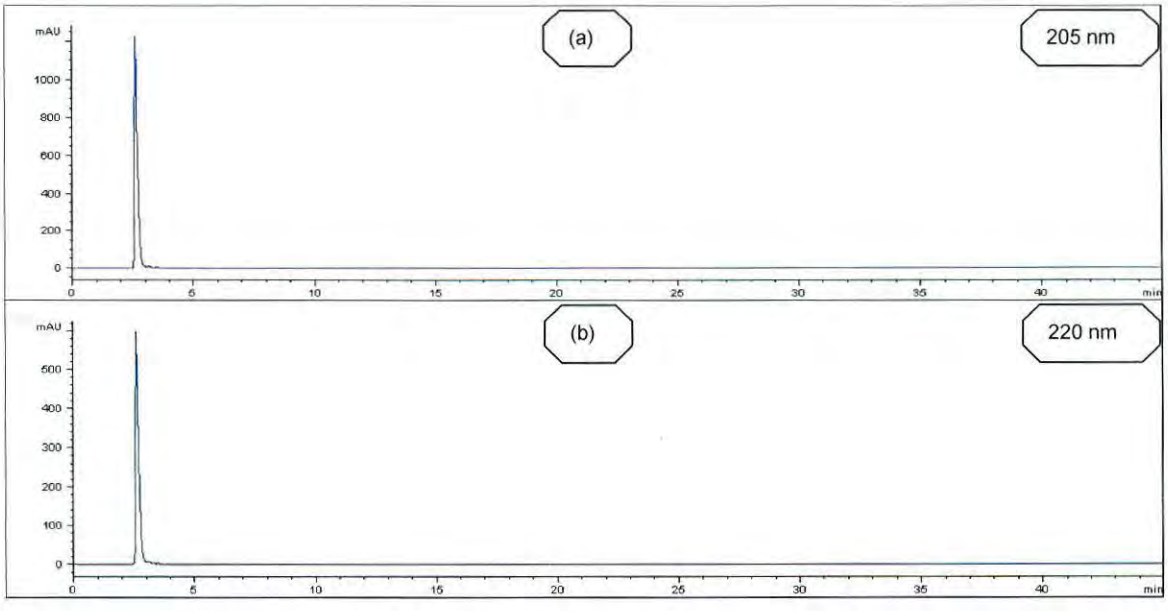

Figure 4.12 Chromatogram obtained with Contrad concentrate® at a concentration of 5% v/v using mobile phase 3, with chromatograms (a) and (b) obtained at wavelengths 205 nm and 220 nm respectively.

Observation of figures 4.10, 4.11 and 4.12, show that Ekon D concentrate® still had a better UV response at 220 nm when compared to Contrad® and LaboClean FT concentrate® which have a better UV response at wavelength 205 nm. The adjustment of the pH of the mobile phase, made a significant difference in the appearance of the chromatograms.

Figure 4.10 of Ekon D concentrate® showed a better peak response at a wavelength of 220 nm as expected, however there were no significant acceptable peaks that could be identified as active unknown peaks. Figure 4.11 of the LaboClean FT concentrate®, showed peaks separation though the peak shapes were not ideal. Figure 4.12 depicting the chromatograms obtained with Contrad concentrate® showed the detection of a peak at approximately 2.5 minutes. This peak showed clear detection at both wavelengths 205 nm and 220 nm with a good peak symmetry. It was also clear that the peak detected at wavelength 205 nm show a better UV response.

Mobile phase 4

The fourth mobile phase was a combination of acetonitrile and a buffer solution in the ratio 25:75. The buffer solution contained 0.02 M of hexanesulphonic acid sodium salt, with the pH of the buffer adjusted to 3.0 with 85% phosphoric acid. A mobile phase flow of 1.0 ml/min was employed with an injection volume of 25 µl, using a µBondapak C_{18} (10 µm) 300 x 3.9 mm column at ambient temperatures and the UV detector set at 205 nm and 220 nm.

Figure 4.13 is a chromatographic representation of Ekon D concentrate® at concentration 1% v/v obtained with mobile phase 4 at 205 nm and 220 nm. These chromatograms showed quantifiable peaks though with a low UV response. The chromatogram obtained with the fourth mobile phase for Ekon D concentrate® was acceptable. The two peaks identified on the chromatogram were used as identification peaks for Ekon D concentrate®.

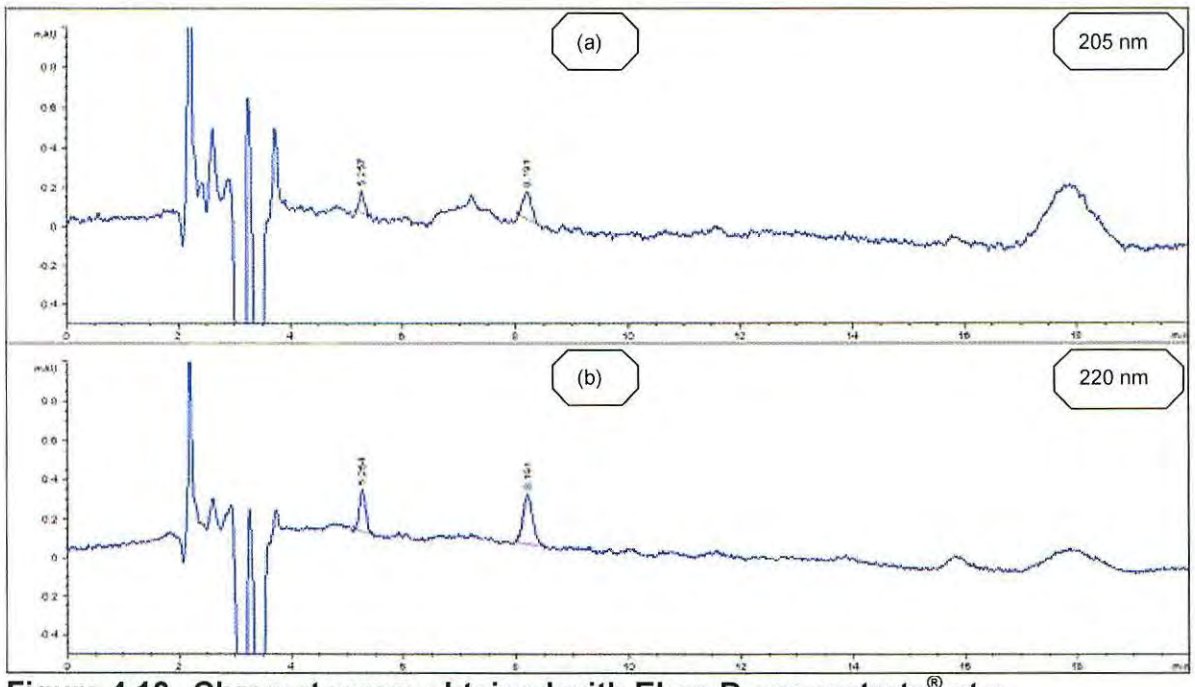

Figure 4.13 Chromatogram obtained with Ekon D concentrate® at a concentration of 1% v/v using mobile phase 4, with chromatograms (a) and (b) obtained at wavelengths 205 nm and 220 nm respectively.

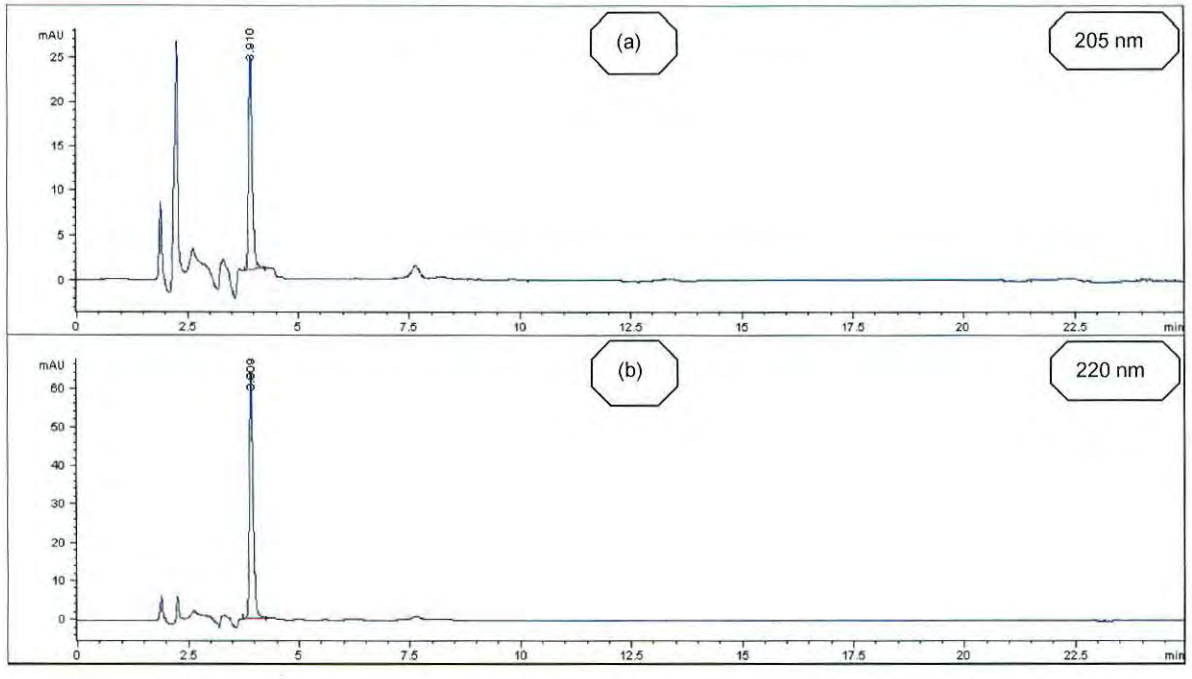

Figure 4.14 Chromatogram obtained with LaboClean FT. concentrate® at a concentration of 1% v/v using mobile phase 4, with chromatograms (a) and (b) obtained at wavelengths 205 nm and 220 nm respectively.

Figure 4.14 is a chromatographic representation of LaboClean FT concentrate® at 1% v/v obtained with mobile phase 4. A quantifiable peak was obtained for LaboClean FT concentrate® when using mobile phase 4 at the specified chromatographic conditions. Contrary to the results obtained with the first three mobile phases, LaboClean FT concentrate® gave a better UV response at wavelength 220 nm rather than at 205 nm as was observed with mobile phases 1, 2 and 3.

Figure 4.15 is a chromatographic representation of Contrad concentrate® obtained with mobile phase 4 at a concentration of 1% v/v. The Contrad concentrate® still gave the most ideal peak shape and a high UV response at wavelength 205 nm when compared with Ekon D and LaboClean FT concentrate®. When observing this figure at wavelengths 205 nm, 212 nm and 220 nm, it is evident that height of the peak increased with a decrease in wavelength. Wavelength 212 nm was only used for trial purposes during method development.

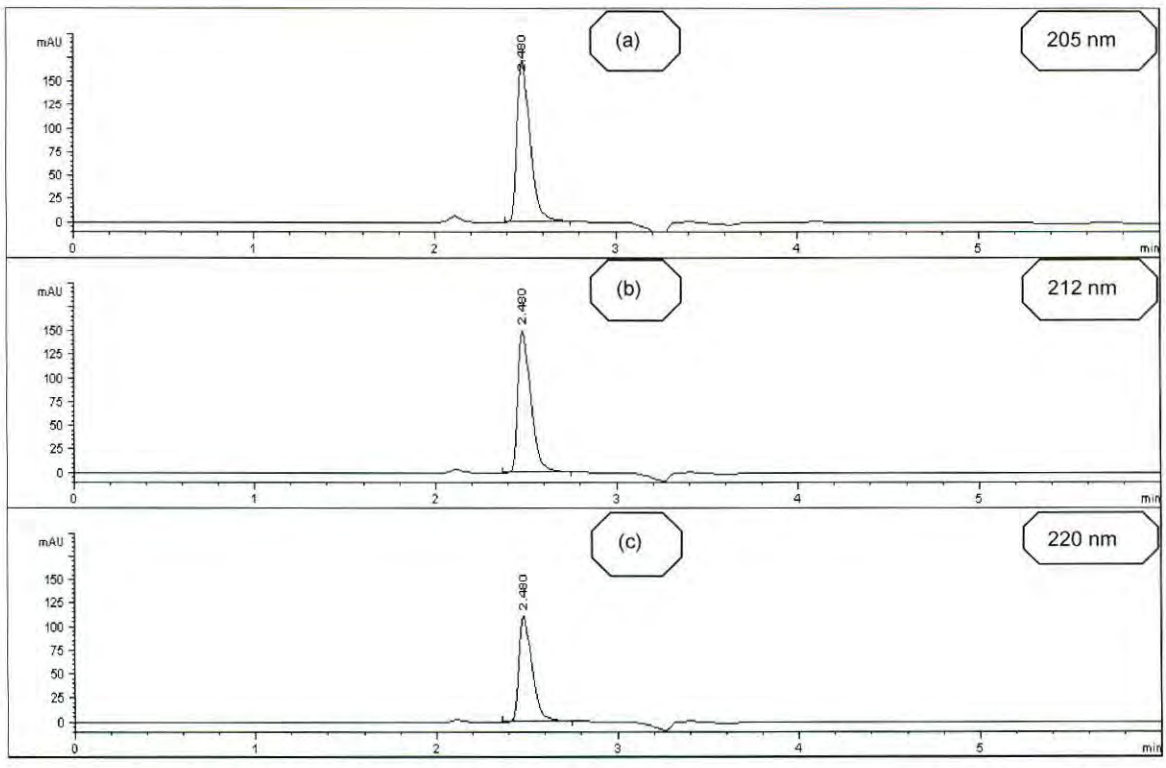

Figure 4.15 Chromatogram obtained with Contrad concentrate® at a concentration of 1% v/v using mobile phase 4, with chromatograms (a), (b) and (c) obtained at wavelengths 205 nm, 212 nm and 220 nm respectively.

4.3.4 Conclusion

With the experimental observations discussed above, a conclusion was drawn on grounds of the reported chromatograms to use mobile phase 4 for investigating the efficiency of the current in-house glassware cleaning protocols in the laboratory of study. For investigating the efficiency of the current in-house glassware cleaning protocol (paragraph 4.4) and validation of the HPLC method for the detection of detergents (chapter 5), only Ekon D® and LaboClean FT concentrate® will be investigated, as they are used on daily basis in the laboratory of study.

4.4 INVESTIGATION OF THE EFFICIENCY OF THE CURRENT IN-HOUSE GLASSWARE CLEANING PROTOCOLS

Investigating the efficiency of the current in-house manual and automated glassware cleaning protocols requires the determination of the limits of operation of the developed HPLC method to ascertain the possibility of accommodating lower cleaning limits (Plasz, 2005). To determine the limits of the developed HPLC method, variable known concentrations of the detergents (Ekon D® and LaboClean FT concentrate®) were prepared.

Table 4.2 in the following page shows the preparation of variable concentrations of Ekon D concentrate ® and LaboClean FT concentrate® to determine the operational limits of the developed HPLC method. The standards were prepared the same way though separately for each detergent.

4.4.1 Chromatographic conditions

Chromatographic conditions established in Table 3.1 were employed.

Table 4.2 Standard preparation for determination of the developed HPLC method operational limits

Determination of the developed HPLC method operational limits	
Standard preparation of Ekon D® and LaboClean FT. concentrate®	
Stock solution	Pipette 5 ml of the detergent to a 100 ml volumetric flask. Add and make up to volume with solvent and mix well.
Dilution A	Pipette 2 ml of stock solution to a 20 ml volumetric flask. Add and make up to volume with solvent and mix well.
Dilution B	Pipette 4 ml of stock solution to a 20 ml volumetric flask. Add and make up to volume with solvent and mix well.
Dilution C	Pipette 6 ml of stock solution to a 20 ml volumetric flask. Add and make up to volume with solvent and mix well.
Dilution D	Pipette 8 ml of stock solution to a 20 ml volumetric flask. Add and make up to volume with solvent and mix well.
Dilution E	Pipette 10 ml of stock solution to a 20 ml volumetric flask. Add and make up to volume with solvent and mix well.

4.4.2 Results for the developed HPLC method operational limits

Figures 4.16, 4.17 and 4.18 are linear regression plots of results obtained from the standard area responses of Ekon D concentrate® peak 1, Ekon D concentrate® peak 2 and LaboClean FT concentrate® peak.

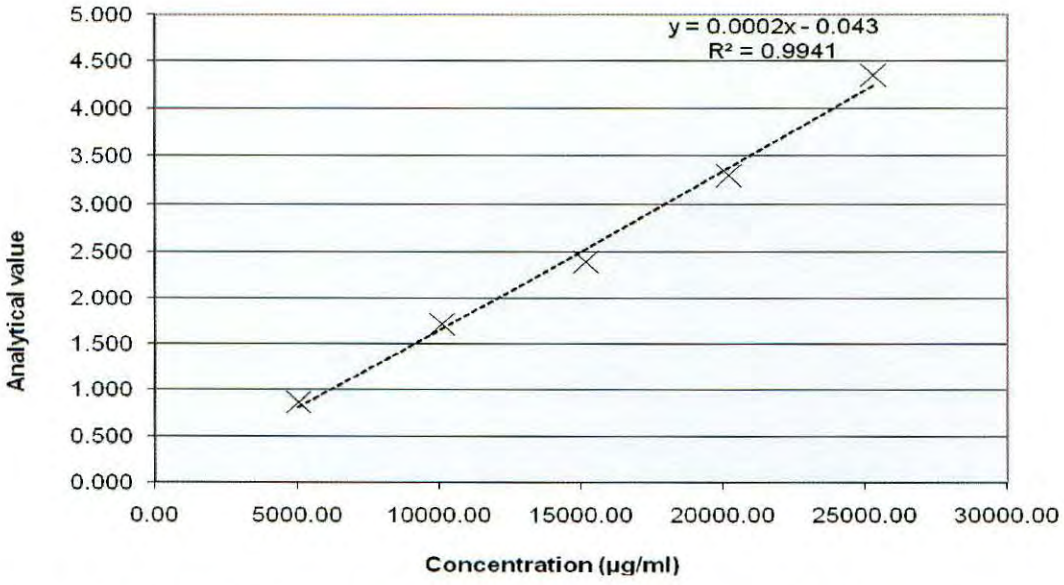

Figure 4.16 Linear plot obtained for Ekon D concentrate® peak 1 area response for the determination of the developed HPLC method operation limits.

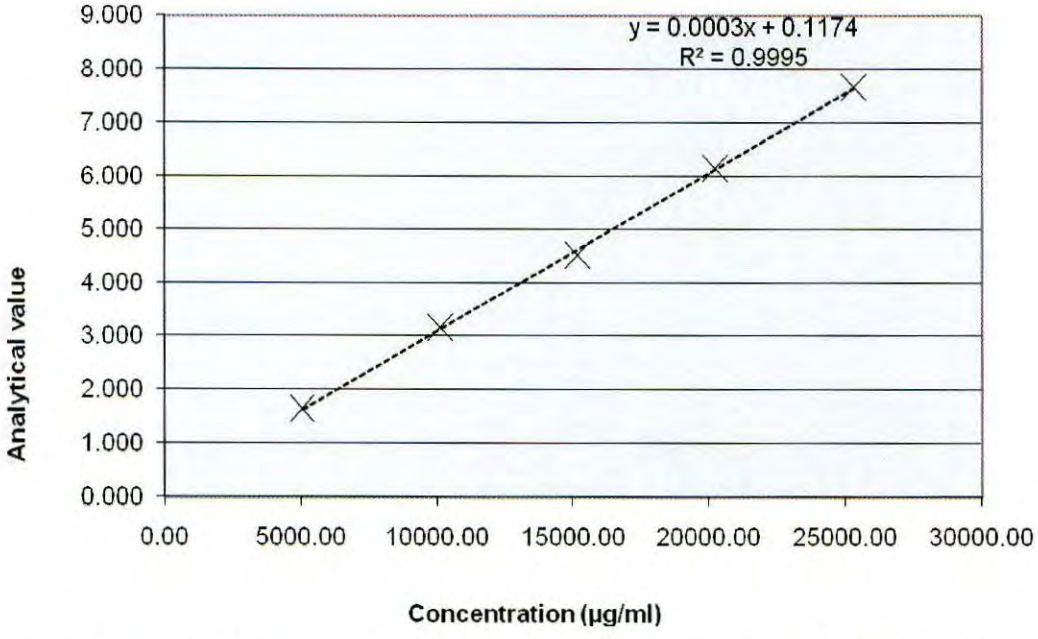

Figure 4.17 Linear plot obtained for Ekon D concentrate® peak 2 area response for the determination of the developed HPLC method operation limits.

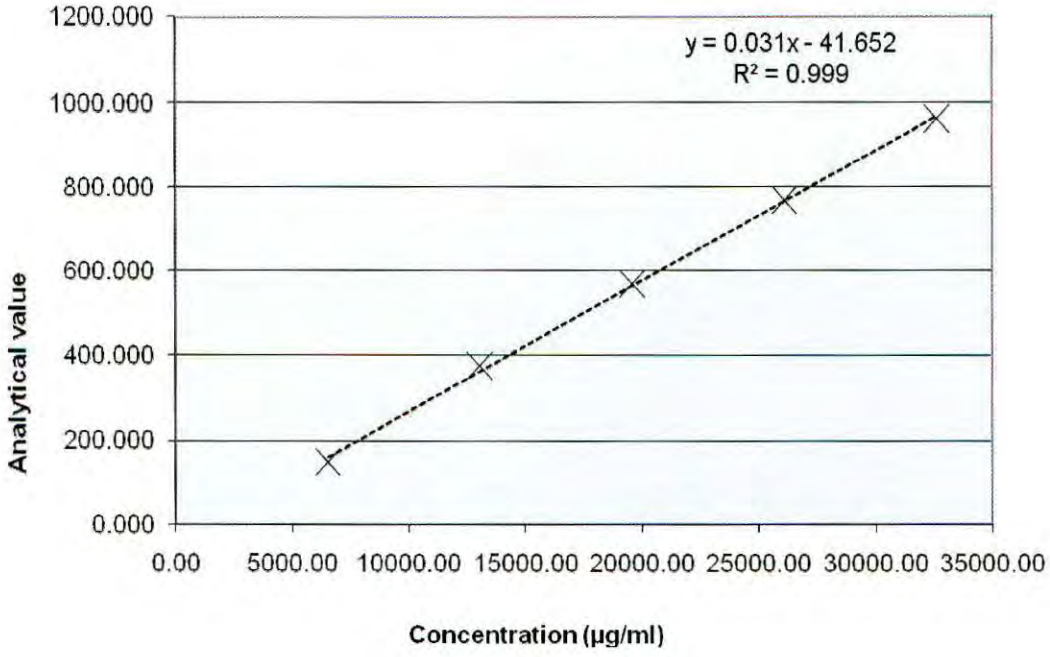

Figure 4.18 Linear plot obtained for LaboClean FT. concentrate® peak area response for the determination of the developed HPLC method operation limits.

Tables 4.3 and 4.4 summarises the results obtained from calculating the peak area responses of the variable standard concentration of Ekon D concentrate® peak 1 and peak 2 respectively. Table 4.5 summarises the results obtained from calculating the area responses of the variable concentration standards of LaboClean FT concentrate® peak.

In the summary report tables the following captions mean the following;

Value (1-5)	= Peak area obtained at the specified concentration
Concentration	= Concentration of the standard solutions in µg/ml
Theoretical concentration	= Concentration of the detergent used as reference
Calculated concentration	= Concentration of the standard per weighed detergent

The captions; system suitability conditions and summary output are explained in section 4.4.3.

Table 4.3 Ekon D concentrate® peak 1 area response summary report obtained for the determination of operational limits of the developed HPLC method

Theoretical 100% concentration (µg/ml)				10120.0					
Analytical values									
Concentration	**% Range**	**Value 1**	**Value 2**	**Value 3**	**Value 4**	**Value 5**	**Average**	**SD**	**% RSD**
5055.0	50.1	0.86	0.87				0.867	0.008	0.946
10110.0	100.1	1.77	1.67	1.74	1.69	1.71	1.71	0.041	2.38
15165.0	150.2	2.40	2.38				2.39	0.015	0.618
20220.0	200.2	3.31	3.29				3.30	0.013	0.394
25275.0	250.3	4.26	4.44				4.35	0.127	2.93

Control Standard							
Theoretical concentration (µg/ml)			**10120.0**	**Calculated concentration (µg/ml)**		10120.0	
Name	**Value**	**Concentration**	**Average**	**SD**	**%RSD**	**% Recovery**	**Uncertainty (x) (µg/ml)**
Control 1	1.62	9792.4	1.66	0.060	3.61	**99.4**	379.3
Control 2	1.70	10292.6					

SUMMARY OUTPUT		**SYTEM SUITABILITY CONDITIONS**		**LOD**	**LOQ**
Regression Statistics		**Response factor 1**	103.4%	2133.6	7112.1
Multiple R	0.997	**Response factor 2**	96.9 %		
R Square	0.994	**USP tailing**	1.01		
Adjusted R Square	0.992	**Theoretical plate count**	9311.0		
Standard Error	0.120	**Capacity**	2.10		
Observations	5	**Resolution**	N/A		

Table 4.4 Ekon D concentrate® peak 2 area response summary report obtained for the determination of operational limits of the developed HPLC method

Theoretical 100% concentration (µg/ml)				10120.0					
Analytical values									
Concentration	**% Range**	**Value 1**	**Value 2**	**Value 3**	**Value 4**	**Value 5**	**Average**	**SD**	**% RSD**
5055.0	50.1	1.65	1.64				1.64	0.005	0.305
10110.0	100.1	3.04	3.25	3.09	3.21	3.15	3.15	0.086	2.74
15165.0	150.2	4.52	4.55				4.54	0.016	0.354
20220.0	200.2	6.11	6.21				6.16	0.017	1.15
25275.0	250.3	7.67	7.64				7.66	0.016	0.206

Control Standard							
Theoretical concentration (µg/ml)			**10120.0**	**Calculated concentration (µg/ml)**		10120.0	
Name	**Value**	**Concentration**	**Average**	**SD**	**%RSD**	**% Recovery**	**Uncertainty (x) (µg/ml)**
Control 1	3.16	10246.3	3.15	0.022	0.700	**100.7**	3.15
Control 2	3.13	10141.5					

SUMMARY OUTPUT		**SYTEM SUITABILITY CONDITIONS**		**LOD**	**LOQ**
Regression Statistics		**Response factor 1**	99.8%	613.9	2046.2
Multiple R	0.001	**Response factor 2**	98.6%		
R Square	0.999	**USP tailing**	0.937		
Adjusted R Square	0.999	**Theoretical plate count**	10944.0		
Standard Error	0.061	**Capacity**	3.81		
Observations	5	**Resolution**	11.0		

Table 4.5 LaboClean FT concentrate® peak area response summary report obtained for the determination of operational limits of the developed HPLC method

Theoretical 100% concentration (µg/ml)				13050.0					
Analytical values									
Concentration	**% Range**	**Value 1**	**Value 2**	**Value 3**	**Value 4**	**Value 5**	**Average**	**SD**	**% RSD**
6525.0	50.1	143.1	152.8				147.9	6.84	4.63
13050.0	100.1	370.5	374.6	377.3	378.8	379.9	376.2	3.76	1.00
19575.0	150.2	568.3	569.3				568.8	0.705	0.124
26100.0	200.2	767.7	769.1				768.4	0.948	0.123
32625.0	250.3	963.3	961.9				962.6	0.958	0.099

Control Standard							
Theoretical concentration (µg/ml)			13050.0	**Calculated concentration (µg/ml)**		13050.0	
Name	**Value**	**Concentration**	**Average**	**SD**	**%RSD**	**% Recovery**	**Uncertainty (x) (µg/ml)**
Control 1	381.0	13642.4	381.4	0.501	0.130	**104.6**	203.7
Control 2	381.7	3665.2					

SUMMARY OUTPUT		**SYTEM SUITABILITY CONDITIONS**		**LOD**	**LOQ**
Regression Statistics		**Response factor 1**	98.7%	1123.6	3745.3
Multiple R	0.999	**Response factor 2**	99.3%		
R Square	0.999	**USP tailing**	1.21		
Adjusted R Square	0.999	**Theoretical plate count**	10913.0		
Standard Error	11.6	**Capacity**	1.31		
Observations	5	**Resolution**	N/A		

4.4.3 HPLC method operational limit results and discussion

4.4.3.1 System suitability

The purpose of system suitability is to define a set of parameters that are a measure prior to each experiment that the system is performing adequately (Lister, 2005). System suitability parameters measured are reported in Tables 4.3, 4.4 and 4.5 for each detergent peak investigated.

- **Peak area repeatability**

Repeatability is the ability of the method to generate similar results for multiple preparations of the same sample (Lister, 2005).

When evaluating Tables 4.3, 4.4 and 4.5 the percentage relative standard deviation (%RSD) calculated for all active peaks at varying concentration is less than 5%. For cleaning validation methods, the acceptance criteria for determining repeatability at a level (concentration) ten times that of the limit of quantitation of the analyte for six replicates is not more than twenty (Lister, 2005).

The %RSD of the Ekon D concentrate® peaks and LaboClean FT concentrate® peak obtained is acceptable for the purpose of the developed HPLC method.

- **Capacity factor (k')**

Capacity factor measures how many times the analyte is retained relative to an unretained component (Ornaf & Dong, 2005). A k' of zero means the component is not retained and elutes with the solvent, whereas a k' between 1 and 20 means the analyte has sufficient opportunity to interact with the stationary phase.

Tables 4.3, 4.4 and 4.5 report k' of more than 1 for the identified active detergent peaks. The values obtained for the k' are large enough and acceptable for the column to provide adequate retention.

- **Tailing factor**

Peak symmetry is an indication of the interaction of the analyte with the stationary phase. A tailing factor is acceptable when it ranges between 0.9 and 1.4, with a value of 1 indicating a perfectly symmetrical peak (Ornaf & Dong, 2005). Peak tailing is typically caused by adsorption or interaction of the analyte with the stationary phase while peak fronting can be caused by column overloading or chemical reaction. Poor symmetry can also indicate a poor column (Agilent, 2004).

The peak tailing results reported for the identified active peaks of the detergents in Tables 4.3, 4.4 and 4.5 range between 0.9 and 1.3. These results indicate an acceptable interaction of the analyte with the column stationary phase. The results also indicate a good column performance.

- **Theoretical plate count**

Plate count or efficiency is an assessment of the column performance (Ornaf & Dong, 2005). The column plate count reported for the identified active peaks of the detergents in Tables 4.3, 4.4 and 4.5 were more than 9000. The column performance is satisfactory for its intended purpose.

- **Resolution (R_s)**

Resolution is the degree of separation of two adjacent analyte peaks, and is defined as the difference in retention times of the two peaks divided by the average peak width (Ornaf & Dong, 2005). The goal of most pharmaceutical HPLC analysis is to achieve baseline separation for all key analytes, with a target resolution value of more than 2.

The resolution value reported in Table 4.4 for Ekon D concentrate® peaks, show acceptable separation between the analyte peaks.

- **Response factor**

The response factor is a measure of the HPLC system performance throughout the entire sequence of analysis. It is determined by calculating the relation ratio as a

percentage of the average repeatability value of the reference standard and the average repeatability value of the control reference standard. Response factor values reported in Tables 4.3, 4.4 and 4.5 for the identified active peaks of the detergents were within the acceptable range of 95%-105% for the cleaning validation type of analysis category.

4.4.3.2 HPLC method limits

Practical detection limit (pDL) and practical quantitation limit (pQL) are two parameters used to establish the operational limits of a method. pDL is based on the signal-to-noise ratio at the lowest level at which the HPLC system can function reproducibly on injections of the standard at a known concentration for the given method (Plasz, 2005). The signal-to-noise ratio of 3:1 is a rule of thumb. pQL is determined at a level 2-5 times the pDL and the repeatability of the standard is determined at this level.

The LOD reported for Ekon D concentrate® peaks 1 and peak 2 in Table 4.3 and 4.4 respectively were different for the same active concentration. This variation is caused by the difference in peak areas. Peak 1 of Ekon D concentrate® has a smaller area when compared to peak 2 of the same active concentration. The calculated LOD of peak 1 of Ekon D concentrate® is approximately 2000 µg/ml whereas the LOD of peak 2 is approximately 620 µg/ml. When evaluating the effectiveness of the glassware cleaning methods, one should keep in mind that peak 2 of Ekon D concentrate® has a better chance of being detected when compared with peak 1 which has a higher detection limit due to the small area size. The calculated LOD for LaboClean FT concentrate® peak reported in Table 4.5 is approximately 1000 µg/ml.

The calculated LOQ of Ekon D concentrate® peak 1 and peak 2 are approximately 7000 µg/ml and 2000 µg/ml respectively, whereas that of LaboClean FT concentrate® peak is approximately 3500 µg/ml. The values obtained for the LOQ of the identified active peaks of the detergents confirm the statement that the LOD is 2-5 times the LOQ of an analyte.

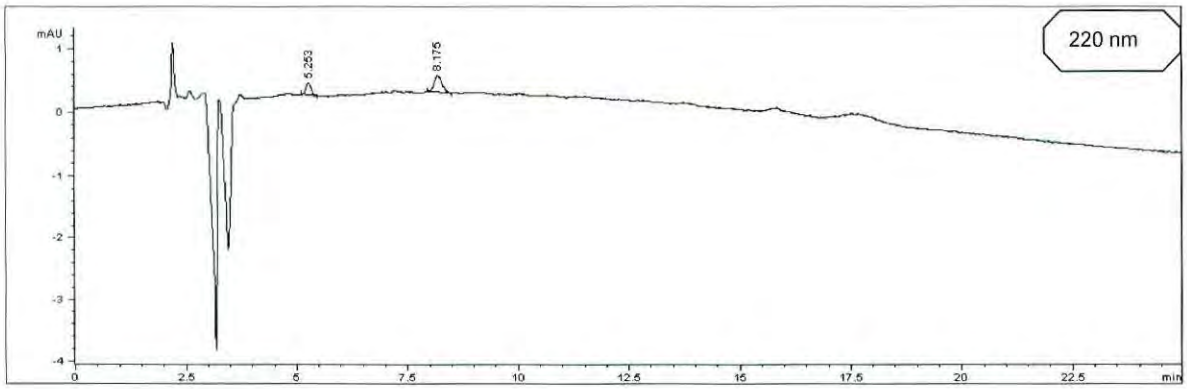

Figure 4.19 Typical chromatogram obtained for Ekon D concentrate® at a concentration of 10 mg/ml with the wavelength set at 220 nm.

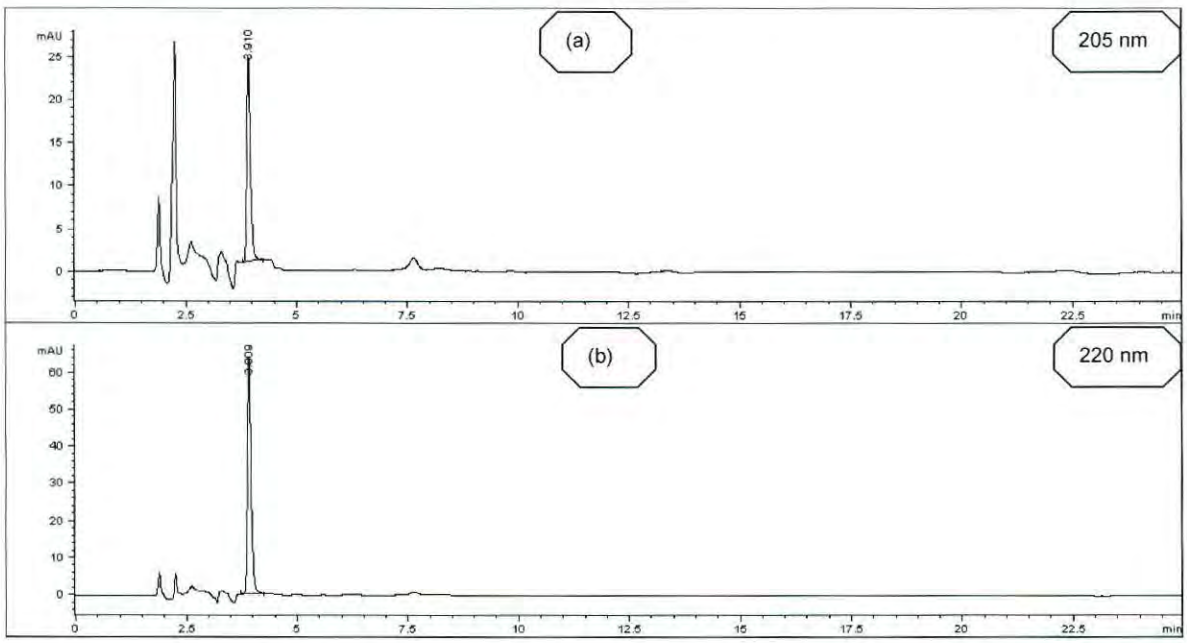

Figure 4.20 Typical chromatogram obtained for LaboClean FT concentrate® at a concentration of 13 mg/ml, with chromatograms (a) and (b) obtained at wavelengths 205 nm and 220 nm respectively.

Figure 4.19 is a typical Ekon D concentrate® chromatogram obtained at a concentration of 10 mg/ml detected with the wavelength set at 220 nm. The first peak of Ekon D

concentrate® elutes at a retention time of approximately 5.3 minutes whiles the second peak elutes at a retention time of approximately 8.2 minutes.

Figure 4.20 is a typical LaboClean FT concentrate® chromatogram obtained at a concentration of 13 mg/ml detected with the wavelength set at 205 nm and 220 nm respectively. The LaboClean FT concentrate® peak elutes at a retention time of approximately 3.9 minutes. Comparing the LaboClean FT concentrate® peak at wavelength 205 nm and 220 nm, the detection of the peak is better at 220 nm wavelength and the baseline is also more stable.

4.4.3.3 Conclusion

The fourth mobile phase and chromatographic conditions could successfully be used to determine the operational limits of the developed HPLC method. A clear distinction of the identified detergent peaks was established with the developed method. Acceptable HPLC system suitability results were obtained in relation to the standards established for cleaning validation guidelines as stated by literature in Plasz (2005).

4.4.4 Cleaning samples

The purpose of the HPLC analysis of cleaning samples is to prove with data that the cleaning procedures work and that the surfaces of the equipment in question are indeed clean. For sampling, the rinse sampling method and swabbing method were employed as discussed in 3.5.4.

4.4.4.1 Rinse vs. swabbing procedures

Standard addition and recovery procedure was employed to help in choosing the best sampling procedure that can be used to investigate the effectiveness of the glassware cleaning procedures.

Two standard and two sample volumetric flasks of the same size containing solution of the same API (active pharmaceutical ingredient) and concentration resulting from analysis performed on daily basis in the laboratory of study, were used to determine the

suitable sampling procedure for this project. The solutions in the flasks were discarded and volumetric flasks were allowed to dry. One dried volumetric flask that contained a standard solution and one that contained a sample solution were sampled using the rinsing procedure and the other two volumetric flasks were sampled using the swabbing procedure. The results of the two procedures were compared.

- **Swabbing procedure results**

Figure 4.21 is the chromatographic representation of the API standard volumetric flask swab. The chromatogram does not show enough quantifiable API recovery.

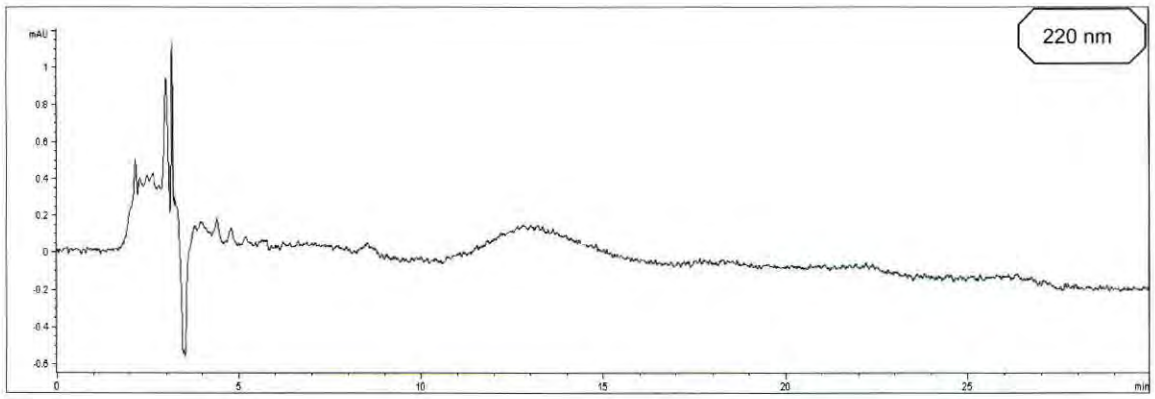

Figure 4.21 Chromatogram obtained from a swab sample of an API Standard volumetric flask, detected at wavelength 220 nm.

Figure 4.22 in the following page is the chromatographic representation of the API sample volumetric flask swab. The swab of the sample volumetric flask showed two quantifiable peaks, with the first peak eluting at retention time 8.10 minutes and the second at retention time 11.43 minutes. The second peak observed on the chromatogram has a wide width. The peak shape is typically an analyte response that is expected when nonspecific chromatographic conditions are used.

When comparing figure 4.21 with figure 4.22 the chromatograms have a similar baseline trend. The peaks identified and quantified in figure 4.22 could only be identified as baseline instability in figure 4.21.

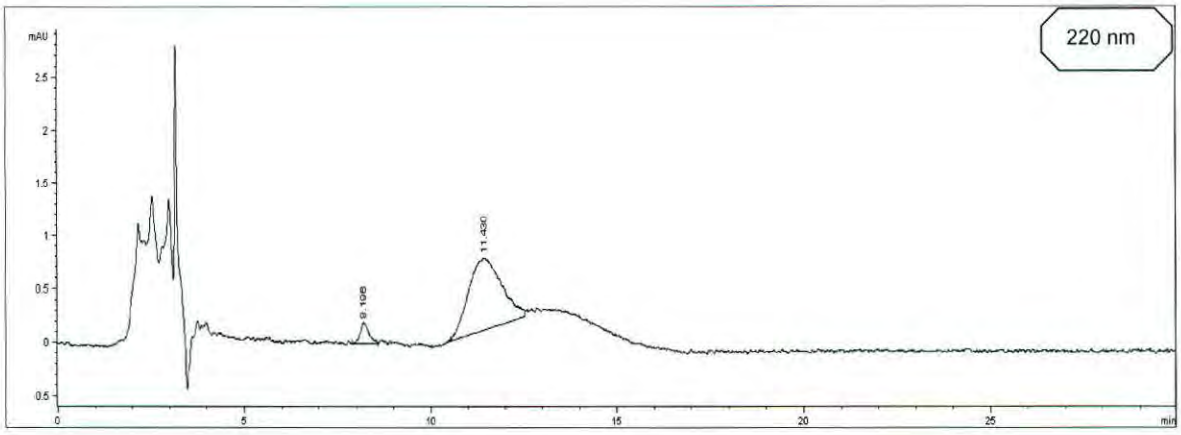

Figure 4.22 Chromatogram obtained from a swab of an API Sample volumetric flask, detected at wavelength 220nm.

Recovery of the API was better with the sample swab as compared to the standard swab. The difference in the recovery of the API might be influenced by the presence of drug associated powders found in the sample that are absent in the standard, as the standard is only composed of the pure form of the API.

A few challenges were encountered with the use of the swabbing sampling procedure. Volumetric flasks range from as little as 5 ml to 5000 ml. Sampling volumetric flasks with a throat swab that is approximately 15 cm long presented a challenge especially with volumetric flasks ranging from 100 ml and more, as the swab was too short for proper sampling. The shape of volumetric flasks also presented a barrier for sampling with a throat swab, as only the neck of the volumetric flasks was accessible for sampling. The bottom bulb of the volumetric flasks that is mostly in contact with drugs being analysed could not be sampled using a throat swab. Another challenge encountered with the swabbing procedure was the detection interferences from sterile swabs.

Figure 4.23 is a chromatographic representation of a sterile swab. At retention time 8.7 a peak resulting from the sterile swab solution could be detected and quantified. This matter added a disadvantage to the reliability of the swabbing sampling procedure, as

the procedure itself added contaminants to the solution in question the results of which contradicts the sampling purpose.

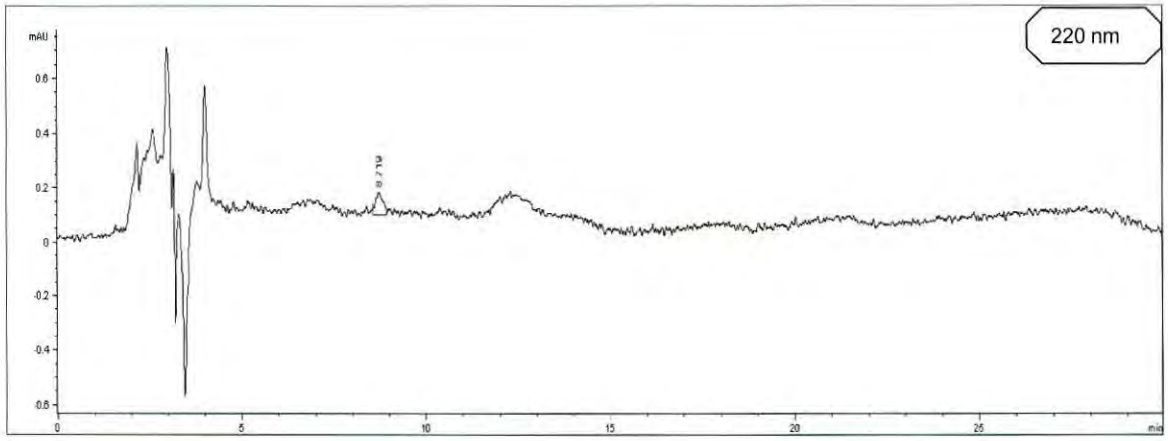

Figure 4.23 Chromatogram obtained from a sterile swab solution, detected at wavelength 220nm.

- **Rinsing procedure**

Figure 4.24 is the chromatographic representation of the API standard volumetric flask rinsate. Figure 4.25 is the chromatographic representation of the API sample volumetric flask rinsate.

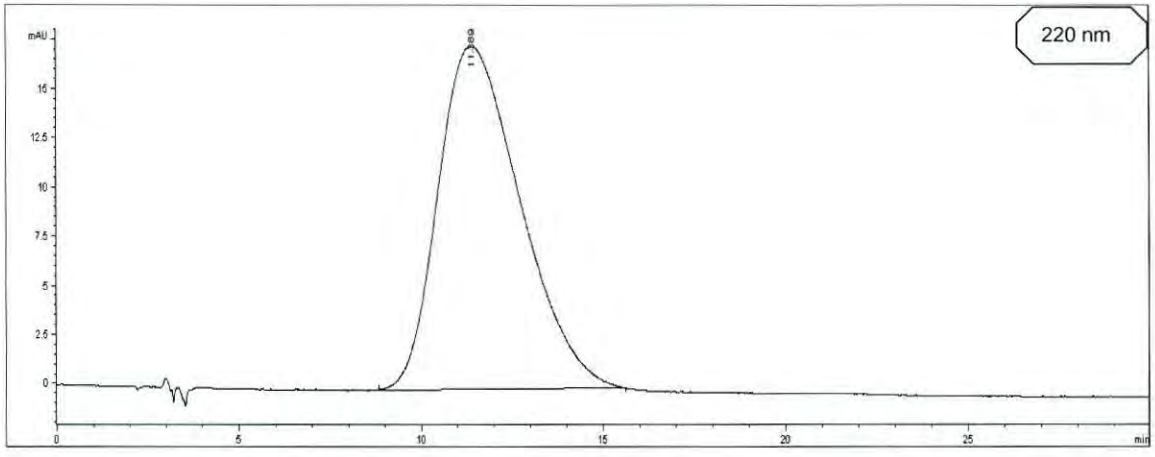

Figure 4.24 Chromatogram obtained from the API standard volumetric flask rinsate, detected at wavelength 220nm.

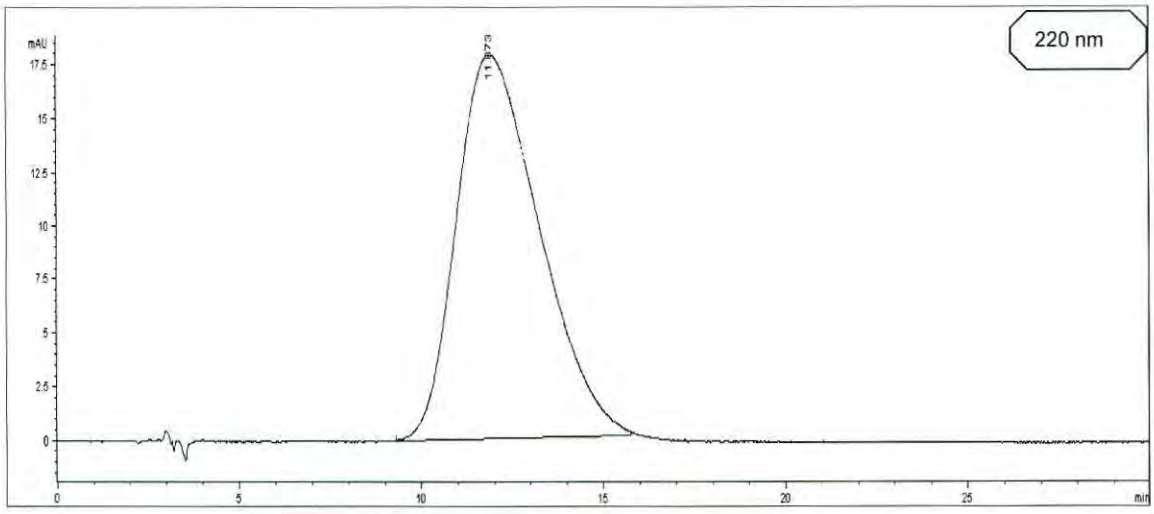

Figure 4.25 Chromatogram obtained from the API sample volumetric flask rinsate, detected at wavelength 220 nm.

The API peak was identified and quantifiable in both figure 4.24 and 4.25. The peak width of the API is not ideal but that is merited by non-specific chromatographic conditions. The recovery of the API from the standard rinsate and that of sample rinsate correlated. The standard rinsate gave an average area response of 2768.3 and the sample rinsate gave a slightly higher average area response of 2850.9 when analysed at a wavelength of 220 nm under the developed method's chromatographic conditions.

4.4.4.1.1 Conclusion

The rinsing sampling procedure proved to be more reliable and suitable for use in this project when compared to the swabbing procedure. Based on the outcome comparison results of the two sampling procedures, the rinsing procedure was used for further analysis in the study.

4.4.4.2 Evaluation of the current in-house cleaning procedure results

With rinse samples, the equipment is rinsed with a known amount of rinsate. Only a

portion is collected and analysed but then concentration is assumed based on the volume of rinsate that one starts with.

Fifty two cleaned 100ml volumetric flasks were randomly selected from the glassware washing area. The sampling procedure was carried out each day for a period of a week, with a clear distinction established between flasks washed with the automatic laboratory glass-washer and flasks manually washed. The glassware was sampled using the rinse sampling procedures (procedure is explained in 3.5.4).

Two HPLC system sequences were set up for the assessment of the current in-house cleaning procedure. The first sequence set up was composed of a total of twelve volumetric flasks, six hand washed and six machine washed. The second sequence set up was composed of a total of forty volumetric flasks, twenty hand washed and twenty machine washed flasks.

Figures 4.26, 4.27 and 4.28 is typical chromatograms of a solvent, a hand washed glassware rinsate sample and a machine washed glassware rinsate sample analysed at 220nm wavelength.

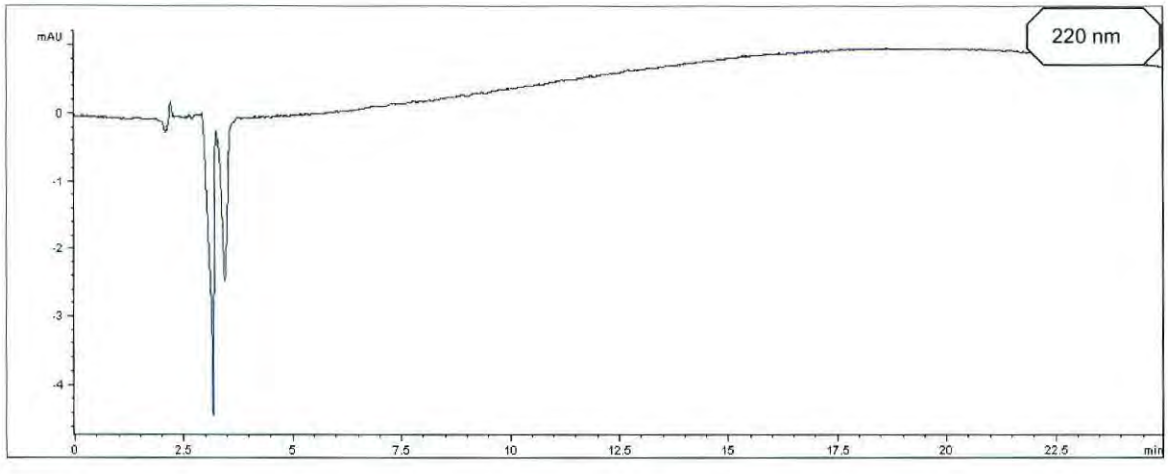

Figure 4.26 A typical chromatogram obtained from injecting the solvent at a wavelength of 220 nm.

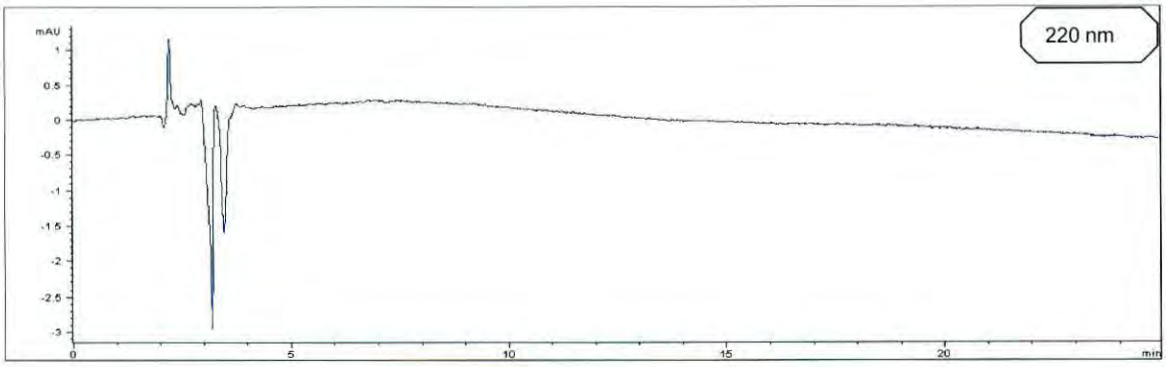

Figure 4.27 A typical chromatogram obtained from the hand washed glassware rinsate, with the wavelength set at 220 nm.

The chromatogram depicted in figure 4.26 show a clean baseline, there was no detection of significant quantifiable peaks. This observation is ideal for the solvent, as there should be no peaks detected for the solvent. Figure 4.27 of the hand washed glassware rinsate also show a clean baseline however at retention time 3.8 there is a small peak that is assumed to be solvent associated. If one closely scrutinises figure 4.19 of Ekon D concentrate® standard the 3.80 peak observed in figure 4.27 is also present in figure 4.19. See figure 4.29 and figure 4.30 for magnified chromatograms. In figure 4.28 there is also a detection of a small peak at retention time of 3.84 which also might be solvent associated because the LaboClean concentrate peak® elutes at a retention time of 3.90 minutes as reported in 4.4.3.2.

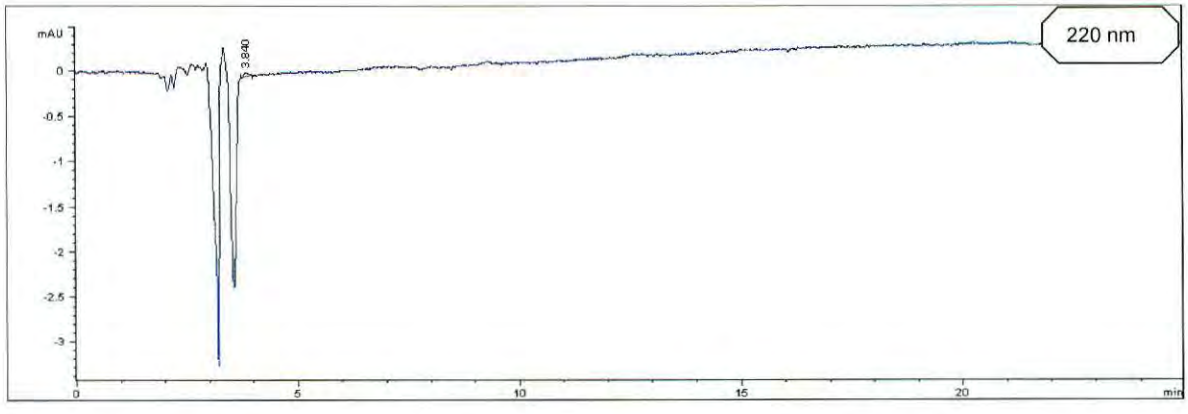

Figure 4.28 A typical chromatogram obtained from the machine washed glassware rinsate, with the wavelength set at 220 nm.

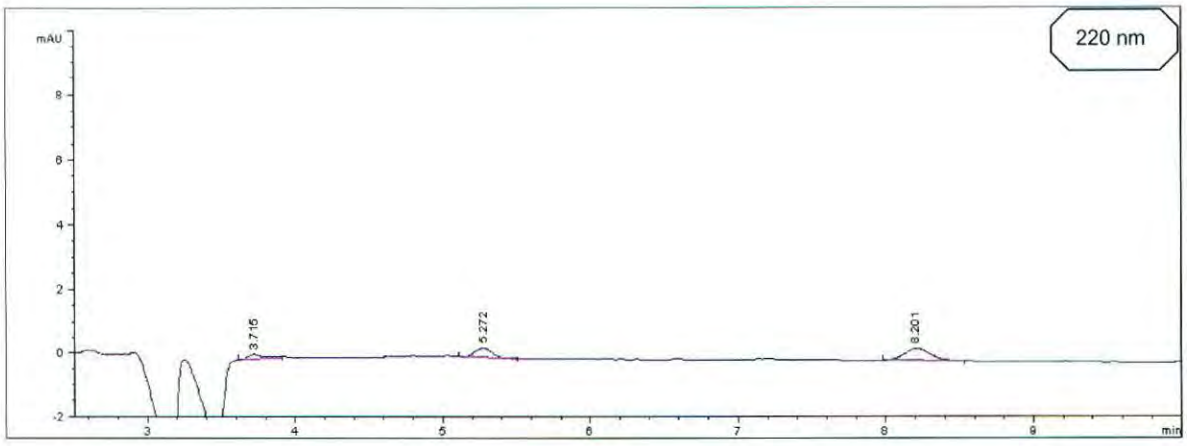

Figure 4.29 Depiction of the solvent associated peak in Ekon D concentrate® standard

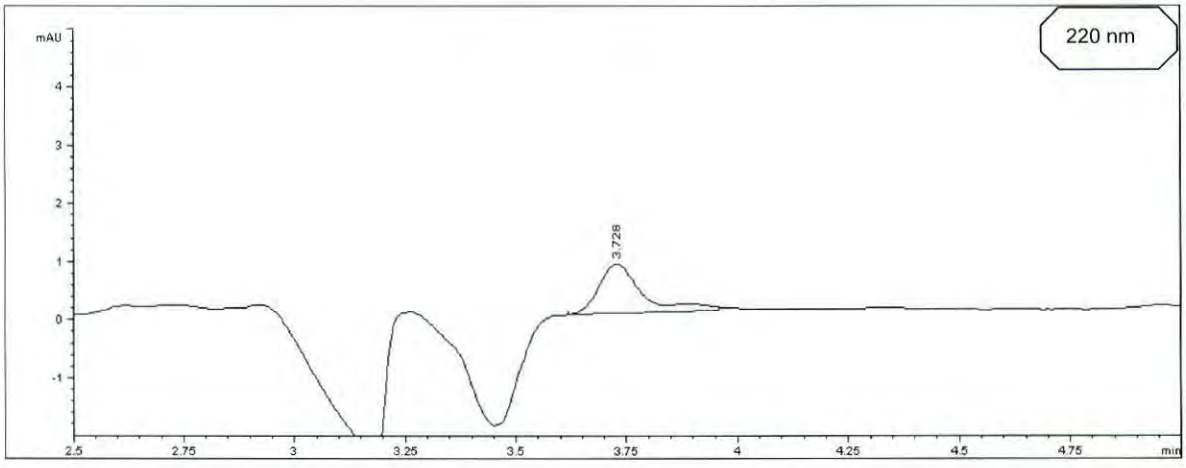

Figure 4.30 Magnification of the solvent associated peak identified in Figures 4.27, 4.28, and 4.29.

- **Hand washed cleaning sample results**

Data generated from the first sequence set up for hand washed volumetric flasks, possible unknown contaminant peaks were detected in two of the six hand washed flasks. In one flask an unknown peak was detected at a retention time of 5.67 minutes and in another flask the unknown contaminant peak was detected at a retention time of 4.95 minutes. The contaminant peaks detected are possibly API associated peaks as

none of the peaks eluted at the retention time matching that of the detergent peaks. In the other four hand washed flasks only the solvent associated peak could be detected, possible contamination peaks were not detected. All flasks used were 100ml volumetric flasks.

In the data generated from the second sequence set up, out of the twenty hand washed volumetric flasks, there were five contaminated flasks. Of the five contaminated flasks, one flask was contaminated with an API (Figures 4.31 and 4.32) and in the other four flasks Ekon D concentrate® residues were detected (Figure 4.33).

- **Machine washed cleaning sample results**

In the data generated from the first sequence set up for machine washed volumetric flasks, one out of six flasks had API related contaminants. The other five flasks were free of contamination. From the second sequence set up for the machine washed volumetric flasks, of the twenty sampled flasks only two flasks had API related contaminants. Detergent traces were not present in any of the machine washed volumetric flasks.

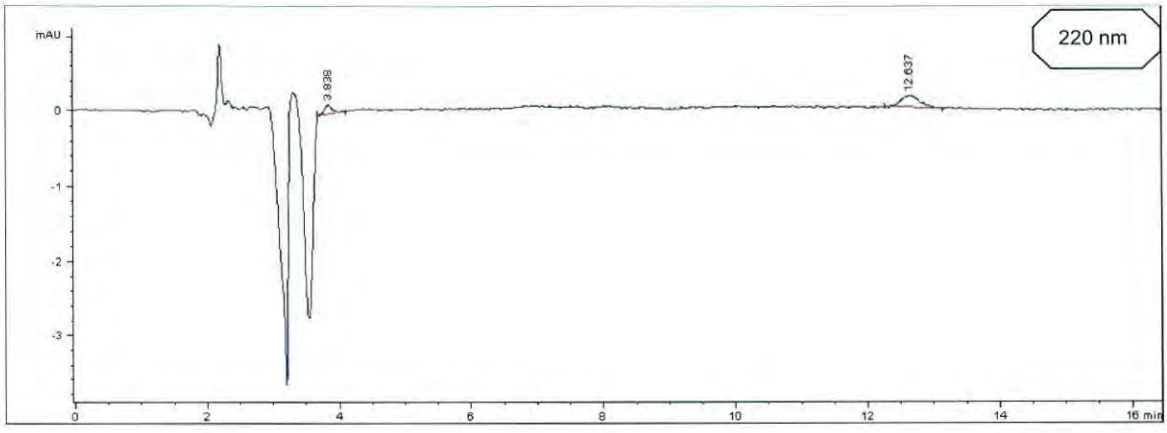

Figure 4.31 A typical chromatogram obtained from the API contaminated volumetric flask rinsate, detected at wavelength 220 nm.

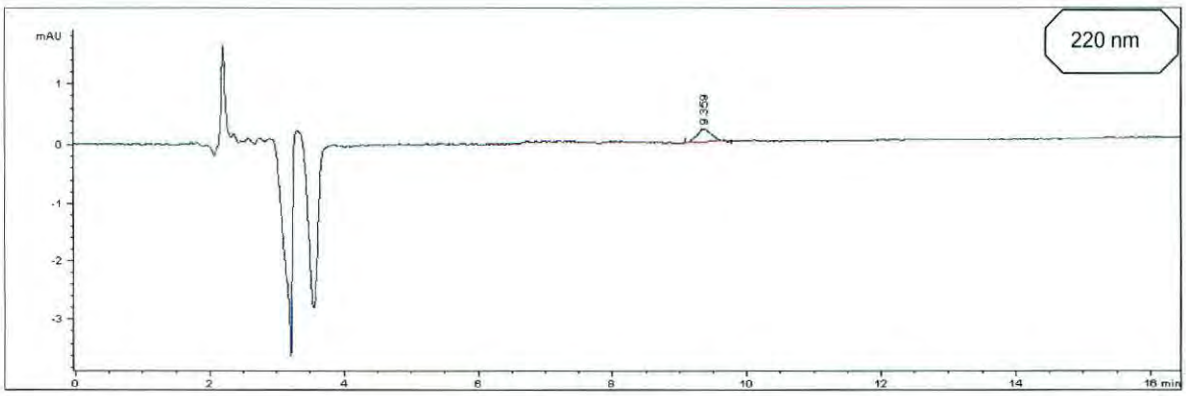

Figure 4.32 A typical chromatogram obtained from the API contaminated volumetric flask rinsate, detected at wavelength 220 nm.

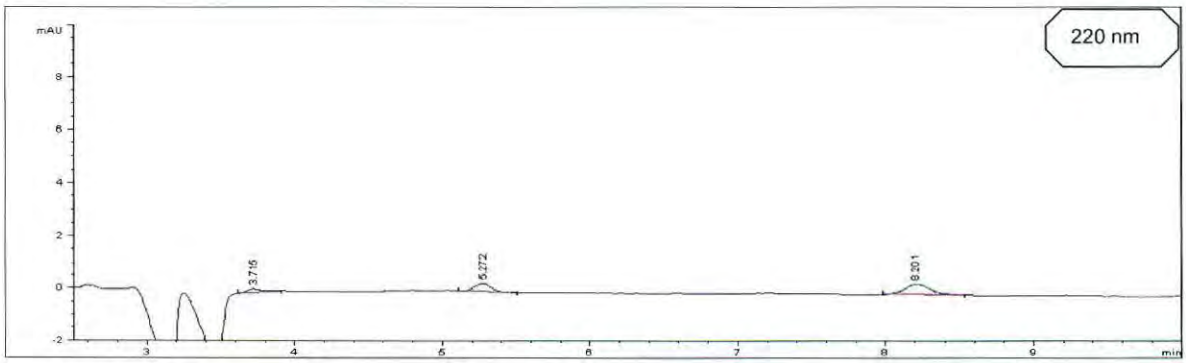

Figure 4.33 A typical chromatogram obtained from a detergent contaminated volumetric flask rinsate, detected at wavelength 220 nm.

- **Control hand washed and machine washed cleaning samples**

Four volumetric flasks of varying sizes were randomly selected from the cleaned laboratory glassware designated cabinet. The cleaned volumetric flasks were thoroughly rinsed with tap water and allowed to dry in the glassware drying oven set at 50°C. One 25 ml, one 50 ml, one 100 ml and one amber 200 ml volumetric flask were sampled using the rinsing sampling procedure described in 3.5.4.

The 25 ml, 50 ml and 100 ml volumetric flasks were free of contaminants, whereas the amber 200 ml volumetric flask was still contaminated with soap and API residues after the thorough rinse.

4.4.4.3 Calculation of the detergent contaminants percentage recovery

Ekon D Concentrate® detergent residues were recovered from some of the hand washed volumetric flasks, as mentioned in 4.4.4.2. The percentage residues recovered from the glassware are reported in Table 4.6.

Table 4.6 Percentage recovery of detergent contaminants

Flask	Std peak 1 average	Std peak 2 average	Concentration peak 1	Concentration peak 2	Percentage recovery	
					Peak 1	Peak 2
1	1.77	3.42	No peak detected	0.050	-	14.8
2	1.77	3.42	0.027	No peak detected	15.7	-
3	1.77	3.42	0.109	No peak detected	62.2	-
4	1.77	3.42	0.046	0.036	26.0	10.7
5 Control	2.86	3.10	No peak detected	0.842	-	271.1

The recovery (g/ml) was calculated using the formula:

$$\frac{\text{Area of sample} \times \text{volume of flask} \times \text{standard concentration(1.012 g)}}{\text{Area of Std} \times \text{volume rinsate} \times \text{Std volume}} = \text{(g/ml)}$$

Recovery (µg/ml) ÷ theoretical concentration of the standard (µg/ml) = % recovery

Where:

Area of sample = the area of the sample peak from the rinsate solution

Area of Std = the area of the standard peak from the standard solution

Volume of flask = the volume of the flask sampled (100 ml)

Volume rinsate = the volume of rinsing solution used to sample the flask (10 ml)

The standard conc. = the standard concentration (grams) considering specific density

Standard volume = the volume to which the standard solution is made up (100 ml)

4.4.4.4 Discussion

Detergent and drug related residues were detected in the hand washed volumetric flasks. No detergent residues were detected on the machine washed glassware however drug related contaminants were detected from the machine washed glassware.

The Ekon D Concentrate® detergent residues percentage recovery calculated in Table 4.6 show the detection of peak 1 below the range established for the LOD and a detection of peak 2 within the LOD/LOQ range for the developed method. These results show that the method is sensitive enough to operate at levels below its predetermined operational limits, adding an advantage to the use of the method in determining the efficiency of the current glassware cleaning protocols in the laboratory of study. One should however take into account the limit of uncertainty for the operation of the method.

The results show serious discrepancies in the cleaning process of the laboratory glassware, especially with the hand washed glassware. Detergent traces were recovered in approximately 16% of the total sampled hand washed glassware and in 13% drug contaminants were recovered. From the machine washed glassware 10% was contaminated with drug residues and none of the flasks was contaminated with soap residues. Recent witnessing of the glassware cleaning procedure has revealed that the current in-house glassware cleaning SOP is not followed correctly for both hand washed and machine washed glassware.

4.5 SUMMARY AND CONCLUSION

The rinse sampling method used to recover drug and detergent traces from glassware proved to be efficient for this study. The detergent residues recovered from the cleaning samples could be successfully quantified in relation to the chosen working detergent standard concentration. Drug associated contaminants recovered from sampled

glassware could not be quantified due to a variety of drugs that are being tested in the laboratory of study however; detection of drug traces was a success.

The development of the HPLC method for the detection of detergent and drug traces recovered from laboratory glassware was a success. Despite the challenges associated with the detection of detergents with low UV chromophore compounds with a specific technique like the HPLC, guidance from the spectrophotometric analyses served the early phase method development stage positively. The HPLC method developed in this study adhered to acceptable performance guidelines specified by Plasz (2005) for cleaning verification purposes.

The efficiency of detergents on glassware exposed to drugs seemed to be relatively acceptable for the intended purpose. Most of the sampled glassware was free from unwanted contaminations to levels regarded acceptable for cleanness and fit for reuse. The detergents used in the laboratory of study for the cleaning of glassware are hence efficient for their intended purpose.

The efficiency of the current in-house glassware cleaning protocol is however a questionable matter. Due to lack of proper implementation of the current in-house glassware cleaning procedure clear improvement suggestions could not be made with regards to the development of an efficient glassware cleaning procedure. Shortcomings were also identified in the current in-house glassware cleaning procedures making it even more difficult to attempt validating the glassware cleaning protocol.

The automated glassware cleaning procedure was observed to be more efficient in the cleaning of laboratory glassware when compared to the manual cleaning procedure. Only drug residues were detected from the glassware washed with the automated glassware cleaning procedure, no detergents residues were detected. Contrary, the manual glassware cleaning procedure contributed detergent traces to glassware. Drug traces were also recovered from the manually cleaned laboratory glassware.

HPLC is a technique of choice for the verification of cleaning programs. HPLC provides a linear, sensitive method for quantitating low levels of contaminant residues. Chromatograms offer a proven track record to support the generated data for HPLC analysis. Nothing is more convincing than a chromatogram with a clean baseline equivalent to the blank control (Plasz, 2005). A well-documented glassware cleaning protocol is worthless if it is not followed and implemented for its intended purpose.