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The Use of Ultrafiltration for Removing Interferences in the Lowry Microassay of Extractable Proteins in Natural Rubber Latex Gloves

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This study evaluates the use of ultrafiltration in removing micro-solutes that might interfere in the Lowry microassay. Reference proteins, glove extracts and 1, 3-diphenylguanidine were subjected to ultrafiltration using a commercial concentrator, which has a membrane filter of 3 kD molecular weight cutoff. Separated fractions were analysed for their reactivity by the MS 1392: 1998 *Lowry mircoassay. Purified reference proteins with molecular weights greater than 3 kD, showed high retentate recovery, which verified the efficacy of separation of the ultrafiltration unit. Micro-solutes were evidently present in both the non-interfering and interfering glove extracts. Irrespective of their original extractable protein (EP) values, the micro-solute content for the non-interfering glove extracts was relatively constant at 28%. This is in contrast with the marked variation observed for the interfering glove extracts. The ultrafiltration process caused an irrecoverable loss of approximately 5% – 6%. The total recovery was comparable for both the non-interfering and interfering glove samples. This indicates that the micro-solutes and/or their interfering components were not detrimental to the filter membrane, causing no obvious degradation of membrane integrity. Some samples may however, necessitate higher number of wash cycles to completely remove the interference and obtain accurate EP values.*

Key words: natural rubber latex; proteins; interference; Lowry microassay; ultrafiltration; natural rubber gloves; bovine serum albumin; RRIM 701

Type I hypersensitivity reactions to natural rubber latex (NRL) proteins are well documented[1–5]. Many methods have been used to quantify the amount of proteins that are extractable from latex products, particularly gloves[6–13]. Among the methods, *ASTM D5712-99/95*[7,8] and *MS 1392:1998*[9] are most widely used and both were adapted from the Lowry assay[14]. Despite the use of an acid precipitation step to purify the proteins, it is well known that some substances interfere with the Lowry assay[15,16]. The interference often causes exceptionally high EP values that contradict findings from the size exclusion high performance liquid chromatography[17] (SE-HPLC) and enzyme linked immunosorbent assay (ELISA) analyses[18].

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This indicates that besides proteins, some micro-solutes, which comprise chemicals used in the compounding of latex, might be concurrently precipitated by the acids, and subsequently interfered with the colour development of the Lowry assay. In the present study, a purification process was first introduced to the test samples prior to the acid precipitation step. The procedure involves ultrafiltration of the sample through a membrane filter using high-speed centrifugation. The efficacy of this method was assessed using reference proteins, glove extracts (non-interfering and interfering types) and 1,3-diphenylguanidine (DPG), which has been reported to cause interference in the Lowry assay[16].

MATERIALS AND METHODS

NRL examination gloves, both powdered and powder-free were obtained from the local manufacturers. The retention efficiency of the ultrafiltration technique was assessed using two reference proteins namely bovine serum albumin (BSA, molecular weight of 67 kD, Sigma Co. Ltd.) and RRIM 701 (purified in-house NRL serum proteins of varying molecular weights from clone RRIM 701). DPG was used to examine the ability of ultra filtration in removing substances that interfere with the Lowry microassay[15, 16, 19–21].

All glove samples were extracted in phosphate buffer saline (PBS 0.01 M, pH 7.2) according to the *MS 1392:1998* procedure[9]. Reference proteins were dissolved in similar PBS buffer. DPG dispersion (25%) was first allowed to settle, following which the upper aqueous phase was removed and further centrifuged at 10 000 × g to clarify the solution. The clarified solution was diluted to varying concentrations using distilled water.

Ultrafiltration of the test samples was carried out according to the operating manual using the *Centriprep*® concentrator (Amicon Inc., Beverly, USA), which has a membrane filter of 3 kD molecular cutoff[22]. *Figure 1* is an illustration of the various components of the concentrator assembly. 10 ml of test sample was first added into the sample container following which, the filtrate collector with a prefabricated hydrophilic membrane was then inserted into the sample container. The entire assembly was next centrifuged at 3000 × g for 2 h at 20°C. The filtrate was collected while the retentate was topped up with PBS to original volume (*i.e.* 10 ml) and ultrafiltered once more under similar conditions for one wash cycle. The wash fraction was collected and the remaining retentate was again topped up to original volume using PBS. The separated filtrate, retentate and wash fractions were next subjected to acid

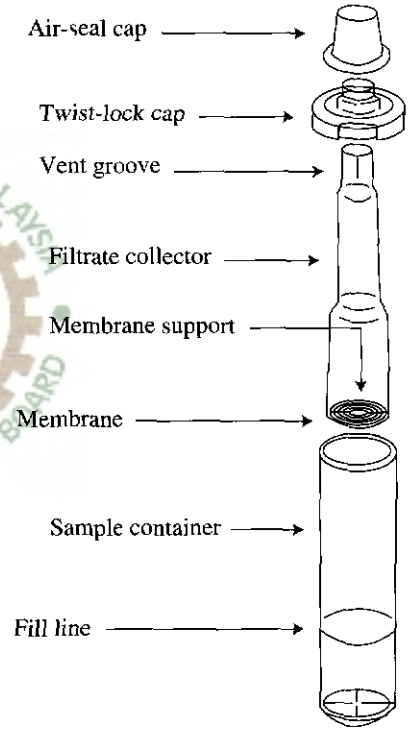

Figure 1. Centriprep® concentrator components[22].

precipitation and Lowry microassay according to the *MS 1392:1998* procedures[9].

RESULTS

Since the micro-solutes comprise both small polypeptides and non-proteinaceous substances, the results in this study are expressed as 'EP or equivalent content' instead of the customary 'EP content' as determined by the Lowry microassay.

Retentate Recovery of the Ultrafiltration Process

The efficacy of the Centriprep® ultrafiltration unit in retaining the larger protein molecules and removing micro-solutes below the molecular weight cutoff of the membrane filter (*i.e.* 3 kD) was examined using the two reference proteins described earlier. *Table 1* shows that when the EP contents of NRL serum proteins (RRIM 701) were above 100 μg/mL, the retentate recovery figures were high (90%–100%). This was similarly observed with high concentrations of BSA (87%–97%). In general, a minimum of 80% retentate recovery was observed at BSA concentrations of approximately 20 μg/ml and above. A further decrease in recovery was apparent when the ultrafiltration process was evaluated at a low protein concentration of 6 μg/ml.

Ultrafiltration of Glove Extracts (Non-interfering Group)

A total of 11 glove extracts, which showed no evidence of interference in the *MS 1392: 1998* Lowry microassay were subjected to ultrafiltration prior to Lowry analysis. *Table 2* shows the EP or equivalent content of the unfiltered (UF), and the retentate (R), filtrate (F) and wash (W) fractions of the glove extracts.

TABLE 1. THE RETENTATE RECOVERY IN THE ULTRAFILTRATION OF REFERENCE PROTEIN SOLUTIONS

Bovine serum albumin (BSA) EP content (μg/mL)			RRIM 701 latex serum protein EP content (μg/mL)		
Unfiltered (UF)	Retentate (R)	Recovery %	Unfiltered (UF)	Retentate (R)	Recovery %
123	109	89	111	111	100
105	91	87	111	102	92
106	92	87	114	110	96
96	93	97	114	111	97
108	98	89	111	103	93
46	41	89	108	97	90
57	48	84	109	103	94
20	16	80			
12	9	75			
9	8	89			
6	2	33			

Without ultrafiltration, the EP content of gloves ranged from approximately 20 μg/g – 1000 μg/g. Subsequent ultrafiltration retained approximately 66% of the original EP values, of which 34% were presumably the filtered micro-solutes. The calculated average for micro-solute content was only 28%, resulting in approximately 6% loss in the micro-solute recovery. Despite the wide range in EP values of the unfiltered samples (approximately 20 μg/g – 1000 μg/g), the percentages of micro-solutes removed were comparable among the glove extracts, giving an average of 28% with a small variation of only ±9%. The total recovery for retentate plus micro solutes was 94%.

Ultrafiltration of Glove Extracts (Interfering Group)

Four glove extracts that showed apparent interferences in the Lowry microassay, were used to examine the extent of sample purification *via* the ultrafiltration process. *Table 3* shows that the retentate recovery was approximately 60% ± 30%; the other 40% were presumably the micro-solutes. The calculated micro-solute contents varied at a substantially broad range (35% ± 37%), depending on the glove samples. The average total recovery was 95% ± 8%.

TABLE 2. EFFECTS OF ULTRAFILTRATION ON THE TOTAL EXTRACTABLE PROTEIN (EP) OR EQUIVALENT CONTENT OF NATURAL RUBBER LATEX GLOVE EXTRACTS

Glove no.	EP or equivalent (μg/g) Unfiltered (UF)	Retentate (R)	Micro-solutes (MS) Filtrate (F)	Wash (W)	% retained (R/UF × 100)	% MS [(F+W)/UF × 100]	% total recovery [(R+MS)/UF × 100]
1	998	733	136	67	73	20	94
2	773	526	98	78	68	23	91
3	437	290	84	36	66	27	94
4	354	225	76	33	64	31	94
5	111	72	27	12	65	35	100
6	77	43	17	8	56	32	88
7	74	48	20	7	65	36	101
8	61	47	3	5	77	13	90
9	56	41	3	7	73	18	91
10	27	18	7	5	67	44	111
11	22	11	4	3	50	32	82
				Mean	66	28	94
				(SD)	(7)	(9)	(7)

TABLE 3. THE EXTRACTABLE PROTEIN (EP) OR EQUIVALENT CONTENTS OF THE VARIOUS FRACTIONS OBTAINED FROM THE ULTRAFILTRATION OF GLOVE EXTRACTS THAT INTERFERED WITH THE LOWRY MICROASSAY

Glove no.	EP or equivalent (μg/g) Unfiltered (UF)	Retentate (R)	Micro-solutes (MS) Filtrate (F)	Wash (W)	% retained (R/UF × 100)	% MS [(F+W)/UF × 100]	% total recovery [(R+MS)/UF × 100]
1	903	724	100	31	80	14	95
2	280	185	44	20	66	22	89
3	302	47	177	96	16	91	106
4	402	299	41	15	74	14	89
				Mean	60	35	95
				(SD)	(30)	(37)	(8)

Ultrafiltration of 1,3-diphenylguanidine (A Known Interfering Agent)

DPG was selected to evaluate the ultrafiltration process because of its noted interfering ability in attenuating the absorbance readings of the Lowry assay[16]. *Table 4* shows that the *UF, R, MS*, and *Total Recovery* results displayed a systematic trend to diminish with reducing concentration of DPG. The extent of filtration also increased with decreasing micro-solute concentrations, as shown by the lower retentate recovery at higher dilution of DPG solutions. There was appreciable discrepancy in the percentage of recovery between the retentate and micro-solute fractions, causing the total recovery to be markedly erratic (60%–137%). The retentate value for 0.75% DPG was markedly low (approximately 2 μg/mL) and far below the 20 μg/mL detection limit of the Lowry microassay[18].

DISCUSSIONS

Certain NRL compounding chemicals are known to react with the Folin reagent and attenuate the absorbance readings, resulting in false positive results in the Lowry microassay[15, 16]. The extent of their interference is not known but their presence will inevitably affect the actual EP values. This study shows that ultrafiltration through the use of a commercial concentrator with an attached membrane filter, is one feasible method in removing the micro-solutes that interfere in the Lowry microassay.

Reference Proteins

The two reference proteins used in this study are materials that have been purified prior to use. BSA, a commercial protein purified by cold alcohol precipitation (Sigma Co. Ltd.,

TABLE 4. THE SEPARATION OF 1,3 DIPHENYLGUANIDINE (DPG) MICRO-SOLUTE BY ULTRAFILTRATION

DPG conc. %	EP or equivalent (μg/ml) Unfiltered (UF)	Retentate (R)	Micro-solutes (MS) Filtrate (F)	Wash (W)	% retained (R/UF × 100)	% MS [(F+W)/UF × 100]	% total recovery [(R+MS)/ UF × 100]
6	135	72	80	33	53	84	137
3	99	51	48	16	52	65	117
1.5	68	23	29	8	34	54	88
0.75	33	2	16	2	6	54	61

catalog number A0281) was selected because it is the protein standard used in the *MS 1392:1998* Lowry microassay. Although the other reference protein RRIM 701 is not used as a standard in the Lowry microassay, it was nevertheless also selected since it represents the myriads of proteins existing in the raw NRL. The RRIM 701 proteins have undergone exhaustive dialysis and subsequently lyophilised prior to use.

In general, the single molecular weight BSA (MW = 67 kD)[22] and varied molecular weight RRIM 701 (molecular weight ranged generally from 14 kD–66 kD)[23] showed high retentate recoveries (*Table 1*). This suggests that the ultrafiltration process did not cause appreciable loss of proteins and was efficient in retaining proteins with molecular weights above the 3 kD cutoff of the membrane filter. Separate experiment in our laboratory showed undetectable readings in the filtrate and wash fractions of the reference proteins. The slightly higher retentate recovery of RRIM 701 in comparison to BSA could be attributed to the heterogeneity of proteins present in the former, which might affect the filterability of the proteins. Poor retentate recovery at low protein concentration is not unexpected since sample concentration is one factor that could affect the solute recovery in the retentate[22]. The greater fluctuation in retentate recovery at low protein concentration is likely attributed to the decreased sensitivity of the Lowry microassay at it approaches the detection limit of 20 μg/mL[18].

NRL Glove Extracts

Unlike the reference proteins described above, all glove extracts showed substantial reduction in the EP values after ultrafiltration. This indicates that micros-solutes were extracted along with the NRL proteins during extraction of the glove samples. Ultrafiltration thereafter evidently removed some micro-solutes. The extent of removal is likely to improve with increasing wash cycles as some of the wash fractions still gave detectable values after one wash cycle. In view of this, it is important that in order to obtain accurate EP values, test samples should be washed repeatedly until the wash fractions no longer produce detectable values.

Regardless of whether or not the glove extracts contain interfering micro-solutes, ultrafiltration will reduce the EP readings of gloves. This may be considered as inappropriate particularly when standard organisations and certifying/regulatory bodies place much emphasis on EP limits as measured by the Lowry methods[24–26]. However, it should be noted that most NRL proteins have molecular weights greater than the 3 kD[23] cutoff used in this ultrafiltration process. A separate study also showed the filtrates to be of extremely low allergenic activities (< 40 AU/mL), suggesting that the removed small polypeptides, if any in the filtered fractions, did not contribute appreciable allergenic activity. It is likely that majority of the micro-solute components comprise non-proteinaceous substances that mimic the interaction of proteins with Lowry reagents. This indicates that it is possible the Lowry assay can indirectly over-estimate the EP content of glove extracts in specific cases.

Irrespective of the unfiltered EP values, non-interfering gloves (*Table 2*) showed an average micro-solute content of 28%, suggesting this to be the general level of micro-solutes present in NRL examination gloves. In contrast, the micro-solute (and indirectly the retentate) contents of interfering gloves varied markedly (*Table 3*). This is not unexpected particularly when interferences are caused by the compounding chemicals. The degree of interference is likely to depend on the amount and type of chemicals present in the glove extracts[15,16], the extent of their interfering ability[15,16] and their chemical solubility in aqueous phase[27] that governs their extractability into the glove extracts. Most importantly, these micro-solute contents were measured using the Lowry microassay, a method not specific for these substances but nevertheless, was observed to produce similar colour development as that observed with NRL proteins. The extent of interference is unlikely to correlate proportionally with the micro-solute concentration when chemical reactions involved are non-specific in nature. In view of this, it is almost impossible to quantitatively gauge the severity of the interference *via* the Lowry assay. Also, the compounding formulations, which are diverse in nature and the proprietary rights of the manufacturers, make it difficult to identify the exact interfering agents. This study showed that false positive results could exceed 500% in EP value (*Sample 3* in *Table 3*). This will undoubtedly result in non-compliance to specifications[24–26]. It is therefore, important to monitor signs of interference in the Lowry microassay and to remove such interferences prior to the protein assay. Notwithstanding the marked variation in the retentate recovery and the micro-solute content, the percentages of total recovery were relatively good and comparable among the interfering glove samples. This suggests that despite the presence of interfering substances, the ultrafiltration process did not cause substantial loss of proteins and micro-solutes. The interfering components of the micro-solutes were apparently not detrimental to the membrane filter, causing no obvious degradation or loss of membrane integrity.

Generally, irrespective of whether the test extracts were from the interfering and non-interfering gloves, the overall irrecoverable loss of ultrafiltration process amounts to approximately 5% – 6%. Besides factors attributed to experimental procedures, irrecoverable loss could also be caused by adsorption onto the membrane filter, which depends on the solute concentration, nature of solute (hydrophilic or hydrophobic), temperature, time of contact with component surfaces, sample composition and pH[22]. Nevertheless, the unexpected consensus in the irrecoverable loss between the two groups of glove samples (interfering and non-interfering) infers that the passage of micro-solutes through the membrane filters was not influenced to a

great extent by the micro-solute composition, at least in the case of glove extracts.

Interfering Micro-solutes

Most compounding ingredients when tested directly in the Lowry microassay generate certain degrees of non-specific reactions[15, 16]. The most apparent of these are the formation of precipitates that increase turbidity of the reaction mixtures and interferences in the colour development of the Folin reagent, \ mostly causing false positive results[15]. DPG was selected for this study because of its strong interference ability, in contrast to several other compounding ingredients, whose interferences were generally below the detection limit of the Lowry assay[16].

DPG is used as a vulcanisation accelerator for polychloroprene latex[28,29], and is also a well-known latex foam stabilizer[28]. The DPG concentrations referred herein are values derived from the dilution of a DPG stock solution, which was obtained from the clarified aqueous phase of the original 25% dispersion. Therefore, except for the original dispersion, the concentrations of other diluted fractions are regarded as the apparent rather than the absolute values.

Ultrafiltration of DPG markedly removed this chemical to below the detection limit of the *MS 1392:1998* Lowry microassay (<20 μg/mL) when tested at an apparent concentration of 0.75%. Although it is obvious that ultrafiltration removed a considerable amount of DPG, the current protocol failed to completely remove DPG tested at other apparent concentrations (1.5% – 6.0%). This suggests that more wash cycles are needed in order to eliminate the interfering substance.

One interesting finding here is that unlike the glove extracts, the percentages of total recovery were found to deviate markedly from the theoretical value of 100%. This is not surprising since the Lowry assay is essentially a protein assay and therefore, non-proteinaceous interfering substances like DPG are not expected to generate results that relate proportionally to their concentrations due to the non-specific nature of the reactions involved. Nevertheless, this should serve as precautionary reminder that interferences by such substances are not measurable quantitatively by the Lowry assay. One way of overcoming this problem is to repeat the wash cycle until no interference is detected in the wash fraction, taking into consideration the risk of losing proteins due to experimental loss, adsorption onto membrane filter and other factors. It is worth noting that DPG is usually not used as a primary accelerator in making NRL gloves. In the event that it is used as a secondary accelerator, it is unlikely that the residual DPG level would exceed 0.75% in the glove extracts. Therefore, ultrafiltration should be adequately effective in removing the residual DPG in the NRL glove extracts, eliminating interference during the subsequent Lowry assay. Other compounding chemicals, such as dithiocarbamates, exist at very low level in glove extracts[30]. If extracted in saline, the levels for such chemicals in NRL gloves are often below the detectable levels of the methods[30].

CONCLUSIONS

In comparison to the purified reference proteins, ultrafiltration of NRL glove extracts showed greater reduction in the Lowry results. This suggests the presence of micro-solutes, which were extracted along with the latex proteins during extraction. In unfiltered glove extracts, some of these micro-solutes could react positively in the Lowry assay, causing an indirect increase in the final protein values. Irrespective of their EP contents, the non-

interfering glove extracts showed relatively similar micro-solute content (average of 28%), contrasting the marked variation shown by the interfering glove samples. The ultrafiltration process also incurred a constant irrecoverable loss of approximate 5%–6% regardless of the type of samples (interfering or non-interfering gloves). Despite the presence of interfering substances, the ultrafiltration process gave an average of 94%–95% total recovery, indicating that there was no substantial loss of proteins and micro-solutes caused by the experimental procedures. This suggests that the components of the micro-solutes were not detrimental to the membrane filter and caused no obvious degradation to the membrane integrity. Although ultrafiltration evidently removes some interfering micro-solutes, it is possible that some samples may necessitate higher number of wash cycles in order to completely remove the interference and obtain accurate EP values.

This study indicates that ultrafiltration is a feasible method to remove micro-solutes that may interfere with the analysis of NRL extractable proteins *via* the Lowry microassay. Although ultrafiltration is quite a lengthy process, taking an average of $2^1/_2$ h – 3 h in addition to the usual Lowry procedures, it is obviously less time consuming compared to other purification procedures such as dialysis. Our laboratory experiences indicate that there are relatively few cases of interfering gloves, most of which were specific to certain manufacturers. Ultrafiltration is a complementary method to the routinely used Lowry microassay, providing an option to remove interfering micro-solutes in order to improve the measurement of extractable proteins in NRL products.

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