UPPSALA
UNIVERSITET

KAHO Sint-Lieven

Department of Women´s and Children´s Health

Biomedical laboratory science program

Bachelor's degree 15 credit

A new High Sensitive Functional Nephelometrical Assay for Assaying C- reactive protein in Serum Based on Phosphocholine Interaction

Hani Alsaadi

Ghent 2013

Supervisor: Prof. Dr. Joris Delanghe

Department of Laboratory Medicine

Ghent University Hospital, Belgium

Abstract

C-reactive protein (CRP) is able to bind phosphocholine in the presence of calcium ions. According to a previous functional property of CRP, we tried to develop an affordable and cheap high sensitive nephelometric CRP assay using soy oil.

Serum samples were measured by Nephelometer BNII (Siemens), by mixing the serum with diluted soy oil emulsion (Intralipid®20%) and Tris-calcium buffer (PH 7.5). The measurement took place after 12 min incubation time at 37°C by measuring the agglutination between CRP and phosphocholine. Results from our automated functional assay were compared with results obtained using an immunoturbidimetric CRP assay.

Results showed a good correlation coefficient for method comparison between functional nephelometric CRP assay and immunoturbidimetric CRP assay, r = 0.895, significance level p <0.0001. The limit of detection for the functional nephelometric CRP assay was 0.1 mg/L. However, the within run % CV values for the functional assay were 6.1 % (20 mg/L), 4.7 % (50 mg/L) and 4.5 % (100 mg/L). The between-run % CV values were 17.6 % (20 mg/L), 18.8 % (50 mg/L), and 11.3 % (100 mg/L).

The new functional nephelometric CRP assay enables high sensitive CRP measurement in serum in the range of 0.1 mg/L to 300 mg/L. The functional assay could be used for veterinary analysis due to the ability to measure CRP according to the functional properties, not the morphological properties which depend on specific antibodies.

Keywords: CRP, intralipid, nephelometer, immunoturbidimetric, agglutination.

INTRODUCTION

C-reactive protein (CRP) is considered to be a member of the pentraxin family of protein which can be found in humans and other animal species. It is known as an acute phase protein which has the ability to rise rapidly as a response to injuries, infections, inflammations or different types of sicknesses. It might also rise following non-inflammatory situations like depression, chronic fatigue, sleep disturbance and aging [1]. CRP was discovered in 1930 at the Rockefeller institute for medical research in a patient who had a pneumococcal infection [2].

CRP is predominantly produced in the liver by the hepatocytes, but additional production has also been reported for alveolar macrophages, coronary artery, peripheral blood mononuclear cells, brain neurons, peripheral blood lymphocytes, adipose tissues, corneal and conjunctival epithelial cells, oral cavity and lung epithelial cells [3]. Liver failure might have an effect on CRP synthesis, and a few drugs, metabolized in the liver, reduce CRP values.

The structure of CRP consists of five identical polypeptide subunits, none covalently bound in a cyclic form. The five polypeptide subunits are able to bind phosphocholine molecules in the presence of calcium ions. Phosphocholine residues can be found in many types of bacteria and most biological cell membranes. It can also bind to some non phosphocholine particles such as ribonucleoprotein [4].

CRP can also activate the classical complement pathway by binding to different types of microorganisms and to the surface of damaged tissue. This binding results in the formation of the CRP phosphocholine complex. The complex is recognized by the C1q protein complex with formation of the C3 convertase enzyme which leads to the activation of the complement and to opsonization and phagocytosis [5].

CRP can rise very quickly during an acute event and also decrease very quickly when the acute event is finished. Interleukin 6 (IL-6) is considered to be the main inducer of CRP. Interleukin 1 (IL-1), complement activation products, glucocorticoids and other factors inhibit CRP production. The half-life of CRP is up to 19 h both under sickness and during normal condition. The concentration values may increase from less than 0.1 mg/L during the normal situation, to more than 400 mg/L during sickness. The synthesis rate of CRP reflects the strength of the pathological progress, and the production completely ceases when there is no pathological event anymore in the body [6].

The CRP concentration is a useful nonspecific biochemical biomarker to screen organic disease, to monitor response to treatment and to detect intercurrent infection in immunocompromised individuals [7].

High levels of CRP might be due to severe infection. The assessment of the CRP value must be compared to the clinical symptoms and other tests. Measurement of CRP value alone cannot provide a definite answer on whether a patient has a disease.

In some types of infections, even low CRP values indicate a serious infection, while values above 30 mg/L indicate a more serious infection. An example of this fact is infections in the urinary tract. A moderate elevation indicates that the infection has spread up to the kidneys. Viral infections such as colds do usually not increase CRP at all, or to only a small extent. However, severe viral infections, such as influenza, can sometimes cause CRP values above 50 mg/L. After an operation the CRP is high and after about one to two weeks, values usually return to normal levels [8].

In other inflammatory diseases, such as rheumatoid arthritis or inflammatory bowel disease, CRP values should be normal if the treatment has a good effect; otherwise if CRP increases as an indication of that additional treatment is needed [9].

CRP is considered to be a trustable inflammatory biomarker due to its ability to measure this acute phase protein in different assays with an acceptable coefficient of variation (CV%). It is also a trustable marker due to its relation to the determination of different diseases such as cardiovascular diseases (CVD). The CVD can be confirmed in relation to the measurement of a high sensitive CRP (hsCRP) assay which is a screening test to measure low concentration of CRP. If the range levels of hsCRP is close to or lower than 0.3 mg/L then it can be rated as a good hsCRP assay with good precision [10]. A slightly elevated CRP can predict future cardiovascular proceedings and coronary heart diseases. That was the main reason why the Centre for Disease Control and American Heart Association proposed using hsCRP assay to detect CRP for patients at intermediate risk of heart coronary disease [11].

CRP can be measured using different methods and different instruments, turbidimetry or nephelometry by an immunoassay being the most common. In the immunoassay the sample consists of a complex that is formed by binding specific antibodies to CRP, the concentration is proportionally related to the amount of formed antigen-antibody complex in the serum of the measured sample during the assay procedure. This method is considered to be rather expensive, particularly for developing countries due to the high costs of test kits. Patients in such countries are not able to pay for CRP analysis because of their low personal budget. Such difficult economic circumstances make sick people suffer because sometimes they are treated with antibiotics depended on suspicion only, not with the help of laboratory inquiry. This was the reason why a recent study tried to determine CRP concentration based on the functional properties of CRP [12]. The functional assay is a very cheap method compared

with the immunoassay and is based on the ability of CRP to bind phosphocholine in the presence of Ca^{2+}. The study was performed on a Cobas 6000 analyzer using bichromatically measurement at 600 and 700 nm. The results were promising as it was possible to determine CRP concentration in the range between 7 mg/L – 400 mg/L. However the method was not sensitive enough for clinical use [12].

The nephelometer is a more sensitive detector of agglutination than other instruments which are based on using turbidimetric methods. This was the main reason to choose Nephelometer BNII for development of a functional assay that could replace the manual functional turbidimetric method. To make the functional Nephelometric method affordable a soy oil emulsions was used, which contains phosphocholine (Intralipid®20%) as a natural and cheap reagent.

The aim of our study was to develop a cheap highly sensitive functional nephelometric assay to determine CRP concentration in serum using Nephelometer. This assay was intended to be used for the clinical purposes, primarily in development countries.

MATERIALS AND METHODS

Samples

Ninety-nine serum samples were obtained from the routine laboratory at the Department of Laboratory Medicine, Ghent University hospital in Belgium. The samples had varying CRP concentrations and were collected after search through the laboratory data system. All samples were coded to protect patients' information. The samples were centrifuged at 2000 *g* for 20 min and were subsequently stored at 4°C for a maximum period of one week.

Triglyceride and cholesterol concentrations were determined for all samples using a Cobas 8000 Modular analyzer series.

Immunoturbidimetric assay for CRP measurement

The particle enhanced immunoturbidimetric assay can be considered as a standard technique for determination of CRP concentrations, and was carried out in the laboratory on a Cobas 8000 (c701) instrument (Roche, Mannheim, Germany). Latex particles coated with monoclonal anti-CRP antibodies are able to bind CRP molecules in serum and form aggregates or immune complexes (antibody - antigen reaction). These immune complexes were determined turbidimetrically by determination of scattered light at a wavelength of 800/570 nm. The assay was standardized by using commercial standards for calibration (Roche, Mannheim, Germany). For measurement of serum samples, two reagents were used. Reagent 1 was a Tris buffer with bovine serum albumin, and Reagent 2 consisted of latex particles coated with anti-CRP obtained of mouse and diluted in glycine buffer (Roche, Mannheim, Germany).

Affinity Chromatography

To make a blank standard to measure the lower limit of detection (LOD) and the limit of quantification (LOQ) for nephelometric assay, affinity chromatography was used to purify a serum pool from CRP molecules. This method operated by using immobilized phosphoryl choline as a beaded agarose (Thermo Fisher Scientific, Rockford, USA). The CRP-depleted serum, used as a blank, was made from seven pooled samples. Ca^{++}-buffer (12.1 g TRIS, 11.1 g $CaCl_2$ and 5.8 g NaCl, pH 8) was used to achieve CRP binding to the phosphoryl choline. EDTA-buffer (37.2 g EDTA, 12.1 g TRIS and 5.8 g NaCl, pH 8) was used to disband the

CRP from the phosphoryl choline. The phosphoryl choline was washed three times with EDTA buffer to release the CRP molecules before repeating the purifying step again. The blank was measured by immunoassay using Cobas 8000 analyzer.

Functional nephelometric CRP assay

Nephelometer

A Nephelometer BII (Siemens, Germany) was used to evaluate concentration of CRP. The turbidity was determined by scattered light employed toward the samples. The light beam used for this purpose was a laser beam. The scattered light of wavelength 840 nm was detected on the nephelometer using a light detector, in an angle between 13° and 24° [14]. The intensity of the measured scattered laser beam was related proportional to the amount of agglutinated particles, which had been formed by binding of CRP particles and phosphocholine lipid in the sample. A calibration curve using known concentrations was used to calculate CRP concentration.

Optimization of assay

Two reagents were used to optimize the assay, reagent 1 and reagent 2. Reagent 1 consisted of a Tris (Hydroxymethyl aminomethane) -calcium chloride buffer. The Tris-Calcium chloride buffer was made in the laboratory (Tris 0,1 mol/L; 0,1 mol/L calcium chloride; pH was adjusted to 7.5 by using hydrochloric acid 1 mol/L). Reagent 2 consisted of Intralipid®20% (Fresenius Kabi, Uppsala, Sweden), was diluted with buffer solution (1:700). Intralipid®20% contains purified soybean oil, purified egg phospholipids, glycerol and water, and pH was adjusted with sodium hydroxide to approximately 8 [15,16].

Performance characteristic

Linearity was assessed in the concentration range between 1 mg/L and 300 mg/L. The linearity was calculated by preparing a serum pool with a high CRP concentration, and then making 14 serial dilution samples made of that serum pool. The concentrations of CRP were measured by immunoassay Cobas 8000 analyzer. The samples were measured 3 times using the Nephelometer, the mean value of these 3 measurements was used to calculate the linearity.

Within run and between run was performed using 3 serum pool levels with CRP concentrations of level 1 was 20 mg/L, level 2 was 50 mg/L and level 3 was 100 mg/L. Within-run (n=10) and between-run (n=7) imprecision was assessed within-run analysis was performed in one run in one day and between-run on 4 days frequently with two analyses each day.

The lower limit of detection (LOD) and the limit of quantification (LOQ) were calculated as the mean value + 3 standard deviation (SD) and as the mean value + 6 SD obtained from 10 measurements for a blank sample, respectively. As blank sample the serum pool was used, consisted of seven samples with a low CRP concentration (<0.6 mg/L).

Final settings

12 minutes protocol

Incubation time for all samples measured on nephelometric assay was 12 minutes. The standards used in the calibration were samples from the routine measured by immunoassay Cobas 8000 analyzer.

Dilution settings

Final protocol using the Nephelometer BNII instrument (Siemens, Germany) was obtained using the following instrument settings: The first step was to dilute 30µL of serum in 175 µL of Reagent 1. In a second step 30 µL of reagent 2 was added. The agglutination was made by Intralipid®20%. Firstly the instrument initiated the turbidimetric start measurement of the solution at time 0 sec where all reagents had mixed; then at the end of time duration of 720 sec the instrument achieved the final turbidimetric measurement. The assay started every time with calibration by using a sample of known CRP concentration with known low triglyceride concentration and known low cholesterol concentration.

Method comparison

This comparison was achieved by using 99 samples with known CRP. All samples were measured by using functional assay on Nephelometer Siemens BII. The comparison took place by measuring all samples again by using immunoassay on Cobas 8000 analyzer. The standard calibrator used for all samples was a sample with a CRP concentration of 38.4 mg/L. Triglyceride concentrations also had been measured to maintain impact of triglyceride on the results.

Statistics

Statistical analysis was performed using MedCalc® (MedCalc Software, Mariakerke, Belgium). We used Box-and-Whisker plots and a Grubbs test (double-sided) to detect outliers for our results. However, Passing-Bablok regression was used to determine results for method comparison of the functional CRP assay and immunoturbidimetric CRP assay.

RESULTS

We observed results according to the final settings of our functional nephelometric assay. Under incubation time of 12 min, the calibrator diluted in 6 points, the final results were graded according to the agglutination in rational way compared to these concentrations.

Performance characteristics

Limit of detection and limit of quantification

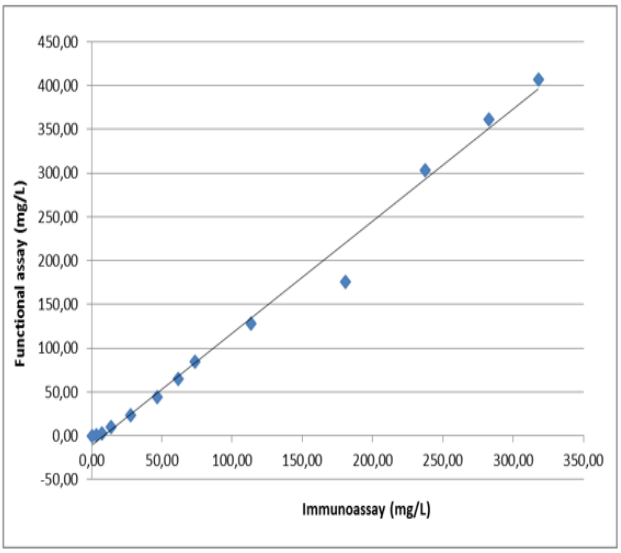
As we wanted to develop a high sensitive assay, we tried to obtain CRP-depleted serum for determination of the limit of detection.

The lower limit of detection of CRP in affinity chromatography depleted serum was 0.0 mg/L with a LOQ value of 0.6 mg/L. The limit of detection and the limit of quantification of the functional assay were 0.1 mg/L and 0.1 mg/L respectively.

Linearity

From the obtained results, a diagram made to show the linearity (figure 1 a, 1 b). The functional assay was found to be linear in the range between 1 mg/L and 350 mg/L, r=0.9941 (Figure 2a). However the linearity was better in the range between 1 mg/L and lower than 200 mg/L, r=0.9949 (Figure 2b).

A



B

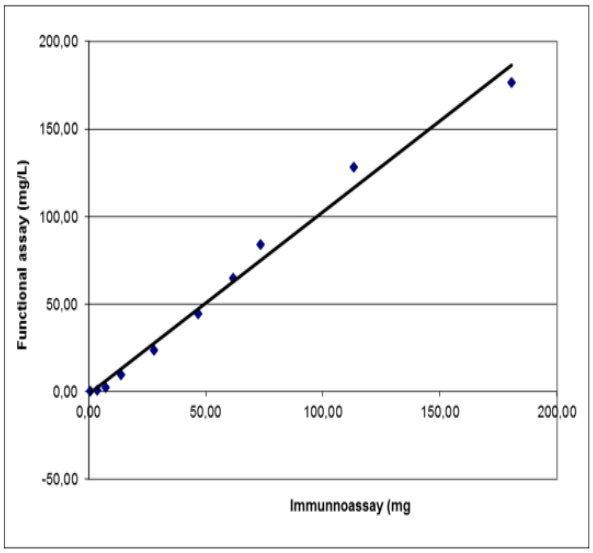


Figure 1 A: Linearity of the functional assay in the range between 1 mg/L and 350 mg/L.
Figure 1 B: Linearity of the functional assay in the range between 1 mg/L and less than 200 mg/L.

Imprecision

Within-run and between-run CV results are presented in table 1. Between the results from the between-run imprecision experiment one outlier for Level 1 could be detected and no outlier for Level 2 and 3.

CRP pools assayed by nephelometry (mg/L)	Within-run CVa (n=10)			Between-run CVa (n=7)		
	Mean (mg/L)	SD (mg/L)	CV %	Mean (mg/L)	SD (mg/L)	CV %
Level 1 (low conc.)	4.5	0.3	6.1	6.4	1.1	17.6
Level 2 (medium. conc.)	21.6	1.0	4.7	26.1	4.9	18.8
Level 3 (high conc.)	65.8	3.0	4.5	68.0	7.7	11.3

Table 1: The CV% of within-run and between run were calculated by dividing the standard deviation SD on the mean for each level measurement.

Method comparison

The results based on the comparison between the immunoassay Cobas 8000 analyzer and functional assay Nephelometry BII. A standard was used of CRP concentration 38.4 mg/L and triglyceride 53 mg/dL.

The results showed a correlation between functional assay and immunoturbidimetric assay, $r=0.8947$, $p<0.0001$. See figure 2.

Two outliers were detected using Box-and-Whisker plots and a Grubbs test (double-sided).

The best correlation was detected at the range lower than 50 mg/L. Y (CRP by functional assay; mg/L) = -3.212 + 1.017x (CRP by immunoturbidimetric assay; mg/L); $r=0.8837$, $p<0.0001$.

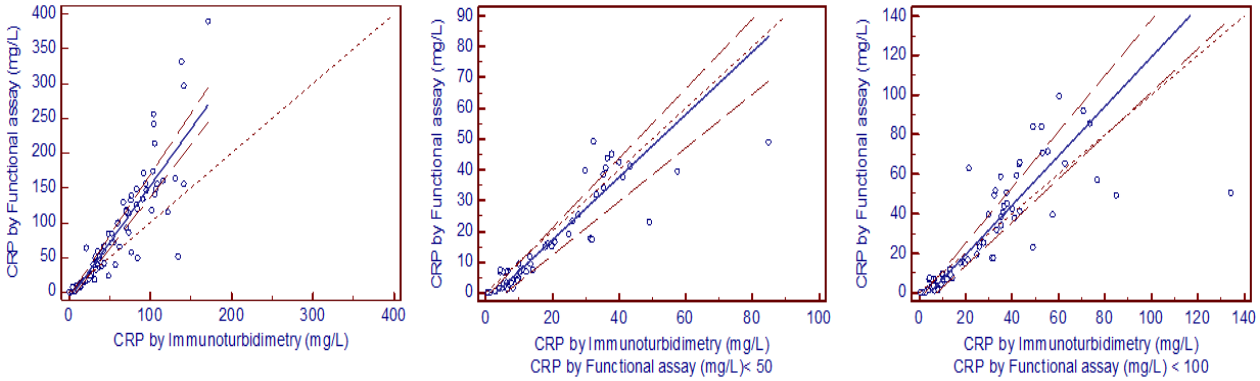


Figure 2: Method comparison between functional CRP assay (y-axis) and immunoturbidimetric assay (x-axis), 99 samples used, 2 outliers detected by Box and Whisker plots and a Grubbs test (double-sided). Diagram to the left shows all samples results, diagram in the middle shows samples results for CRP lower than 50 mg/L, and diagram to the right shows only smples results for CRP lower than 100 mg/L.

However Triglyceride impact on our functional CRP assay was calculated statistically and results showed at table 2. No significant effect on our results.

		Functional CRP assay (mg/L)	**Immunoassay (mg/L)**	
Triglyceride conc.(mg/L)	**Samples**	**Medel ± SD**	**Medel ± SD**	
0-49	3	28,4 ± 31	17,6 ± 9,9	p = 1
50-99	25	94,9 ± 108,1	66 ± 59,4	p = 0,273
100-149	34	73,8 ± 100,8	49,8 ± 52,3	p = 0,849
150-199	20	68,8 ± 73,9	50,5 ± 38,1	p = 0,695
200-249	13	94,7 ± 98,5	63,5 ± 43,5	p = 0,679
250-299	4	139,9 ± 102,2	81,9 ± 54,3	p = 1

Table 2: Impact of triglycerides on results.

DISCUSSION

The initial aim of the study was to obtain good results to measure CRP in different samples by using nephelometer. In order to reach our goal, we examined specific incubation time settings of the assay and we found that 12 minutes incubation was the finest. We used the start dilution of samples to 1:100, and it gave virtuous results to overcome the high dose hook effect. The probability of interaction between CRP molecules and triglyceride molecules were tested. Impact of triglyceride on our assay was calculated statistically, which showed no significant effect.

We were able to reach our aim to develop the functional assay to measure hsCRP nephelometrically by using automatic method instead of manual method [12]. Our nephelometric functional assay was highly sensitive with lower limit of detection of 0.1 mg/L while the manual functional assay had lower limit of detection of only 7 mg/L and the immunoturbidimetric assay 1 mg/L. The BNII Nephelometer has the ability to detect different concentration ranges of samples because Nephelometer has a dilution station which is able to make pre-dilution of a broad spectrum (from 1:1 to 1:2000). Therefore we were able to avoid changing assay program while using different concentrations.

We were able to measure CRP up to 1 mg/L. Meanwhile, our nephelometric functional assay allows determination of low and high ranges of CRP [17]. As a hsCRP assay the main group for prediction disease was the cardiovascular group, which classified to 3 subgroups, low, moderate and high risk, the hsCRP concentration for these subgroups were lower than 1 mg/L, 1 to 3 mg/L and more than 3 mg/L respectively [18].

We calculated the observed imprecision CV results of our nephelometric functional assay and we got less than 6.5 % for the within run imprecision and less than 19 % for the between run

imprecision. Though these results were higher compared with a study of Dominici *et al*. where within-run imprecision for immunonehphelometric assay was around 4 % and within-run imprecision for immunoturbidimetric assay was around 2 % [19]. Results of imprecision CV of our assay were higher than manual functional assay, but in general the functional nephelometric CRP assay has acceptable values according to the results from imprecision criteria based on biological variation where the imprecision <21.1 % [20][1].

Our results showed a good agreement in correlation between the functional nephelometric assay and the immunoturbidimetric assay for assaying CRP in range from 0.1 mg/L and 50 mg/L. The correlation was less good for a broader concentration range from 0.1 to 200 mg/L. The functional nephelometric assay showed a linear curve in the range between 0.1 to 350 mg/L, and the best linearity can be shown between 0.1 and 200 mg/L. We think that the differences in CRP results between the functional nephelometric assay and the immunoturbidimetric assay can be due to the genetic polymorphism of CRP. The measurement of CRP using immunoassay can lead to false negative results, because it can be affected by epitopic variation. However, it can be difficult to phenotype CRP depending on the morphologic properties because of genetic polymorphism [21]. Therefore, the functional assay has an advantage because it is able to determine CRP with different human morphologic shapes or even if it belongs to animals, because the assay was based on the ability to bind to phosphocholine in the presence of Ca^{+2}.

[1] www.westgard.com/biodatabase1.htm. Desirable specifications for total error, imprecision, and bias, derived from intra- and inter-individual biologic variation. Update 2012. 2013-05-02

For veterinary purpose the common method is to measure CRP by using specific monoclonal or polyclonal antibodies. The disadvantage or the problem in this case is to produce and use of different monoclonal or polyclonal antibodies due to variation of species, which might be not easy to produce such variable spectrum of antibodies for laboratory use. It might lead to a variable specificity and sensitivity of detection. Instead, the functional assay can be used with all species without necessitating for producing antibodies, and a recent study shows that it has a good result compared with the immunoassay [13].

We used Intralipid®20% emulsion as a cheap reagent because we were looking to achieve affordable cheap assay which might be suitable for district hospitals of development countries. The size of intralipid particles (diameter about 0.78 µL) make them suitable for turbidimetric analysis and the particle dimensions can be detected at the wavelength of visible light [22,23,24].

Our study provides experimental evidence for an alternative method to measure CRP in serum based on functional properties. This method uses a nephelometer. It was tested here to be used for clinical purposes and we verified the method to be highly sensitive and cheaper than conventional methods.

ACKNOWLEDGMENT

I would like to thank my supervisor professor Joris Delanghe for his advices and feedback. Special thanks for all wonderful staff at the Department of Laboratory Medicine, Ghent University hospital in Belgium for their kindness, hostility and support during my project period. I would also like to thank Stefanie Drieghe for her help and support.

REFERENCES

[1] Aziz N., Fahey J.L., Detels R., Butch A.W. Analytical performance of a highly sensitive C-reactive protein–based immunoassay and effects of laboratory variables on levels of protein in blood. Clin Diagnos Lab Immunol. 2003;10:652-7.

[2] Tillett W.S., Francis T. Serological reactions in pneumonia with a nonprotein somatic fraction of Pneumococcus. J Exp Med. 1930;52:561-71.

[3] Lu Q., Jin L. Human gingiva is another site of C-reactive protein formation. J Clin Periodontol. 2010;37(9):789-796.

[4] Volanakis J.E. Human C-reactive protein: expression, structure, and function. Mol Immunol. 2001;38:189-97.

[5] Mold C., Gewurz H., Du Clos T.W. Regulation of complement activation by C-reactive protein. Immunopharmacology. 1999;42:23-30.

[6] Pepys M.B, Hirschfield G.M. C-reactive protein: a critical update. J Clin Invest. 2003;111:1805-12.

[7] Dandona P. Effects of antidiabetic and antihyperlipidemic agents on C-reactive protein. Mayo Clin Proc. 2008;83(3):333-42.

[8] Ablij H.C., Meinders A.E. C-reactive protein: history and revival. Eur J Intern Med. 2002;13:412-22.

[9] Volanakis, J.E., and Wirtz, K.W.A. Interaction of C-reactive protein with artificial phosphatidylcholine bilayers. Nature. 1979;281:155-157.

[10] Pearson T.A. Markers of Inflammation and Cardiovascular Disease: Application to Clinical and Public Health Practice. Circulation. 2003;107(3): 499–511.

[11] Black S., Kushner I., Samols D. C-reactive protein. J Biol Chem. 2004;279:48487-90.

[12] Tugirimana P.L. A new turbidimetric method for assaying serum C-reactive protein based on phosphocholine interaction. Clin Chem Lab Med. 2009;47:17-22.

[13] Tugirimana P.L. A functional turbidimetric method to determine C-reactive protein in horses. J Vet Diagn Invest. 2011;23:308-311.

[14] Christopeit T., Gossas T., Danielson U.H. Characterization of Ca2+ and phosphocholine interactions with C-reactive protein. Anal Biochem. 2009;391:39-44.

[15] Rowe I.R., Soutar A.K., Pepys M.B. Agglutination of intravenous lipid emulsion ('Intralipid') and plasma lipoproteins by C-reactive protein. Clin Exp Immunol. 1986;66: 241-7.

[16] Tuckwell K.R. Intralipid. Pharmacia and UpJohn product information. MIMS Annual. 1997;19:1110-1.

[17] Sodini I., Morin P., Olabi A., Jiminez-Flores R. Compositional and functional properties of buttermilk: a comparison between sweet, sour and whey buttermilk. J Diary Sci. 2006;99:525–36.

[18] Ridker P.M., Danielson E., Fonseca F.A.H, Genest J., Gotto A.M., Kastelein J.J.P. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med. 2008;359:2195-207.

[19] Dominici R., Luraschi P., Franzini C. Measurement of Creactive protein: two high sensitivity methods compared. J Clin Lab Anal. 2004;18:280–4.

[20] Ricos C., Alvarez V., Cava F., Garcı´a-Lario J.V., Herna´ ndez A., Jime´ nez C.V. Current databases on biological variation: pros, cons and progress. Scand J Clin Lab Invest. 1999;59:491–500.

[21] Elliott P., Chambers J.C., Zhang W., Clarke R., Hopewell J.C., Peden J.F. Genetic loci associated with C-reactive protein levels and risk of coronary heart disease. J Am Med Assoc. 2009;302:37–48.

[22] Michels R., Foschum F., Kienle A. Optical properties of fat emulsions. Opt Express. 2008;16:5907–25.

[23] Bordier C., Andraud C., Charron E., Lafait J., Anastasiadou M., De Martino A. Illustration of a bimodal system in Intralipid-20% by polarized light scattering: experiments and modeling. Appl Phys A. 2009;94:347–55.

[24] Van Staveren H.J., Moes C.J., van Marie J., Prahl S.A., van Gemert M.J. Light scattering in Intralipid-10% in the wavelength range of 400–1100 nm. Appl Opt. 1991;30:4507–14.