Dynamic calibration method for ultrasonic coagulometry

B. Voleišienė, A. Voleišis, R. Kažys. L. Mažeika, R. Šliteris

Prof. K. Baršauskas Ultrasound Institute,

Kaunas University of Technology

Introduction

Blood represents one of the most complex biochemical systems in living organism. Various components play integral roles in several life functions. Numerous studies have been carried out to acquire a more understanding of the properties of the blood coagulation process and increase the capability to diagnose various diseases.

Whole blood test systems, manufactured by different companies and operating on different principles, often give results of limited comparability when testing identical samples of blood. The calibration issue is an important one that should be the subject of a standardisation effort. Calibration of whole blood clotting assays is achieved through a variety of approaches. Consequently, test results between different devices may have limited comparability as well [1].

Popular Sonoclot Coagulation & Platelet Function Analyser (DP-2951) is one of devices for measuring coagulation and platelet function in whole blood or plasma. It measures changes in the viscoelastic properties of the forming clot. Calibration procedures of the Sonoclot analyser involves either a reference viscosity oil or reference plasma (lyophilised animal plasma) [2].

Ultrasonic methods for investigation of whole blood coagulation are based on different principles: spectroscopy [3, 4], measurement of velocity [5-7], attenuation [8], impedance [9] or scattering in whole blood [10]. There is no information about their standardisation while ultrasonic tissue mimicking phantoms are investigated and accepted [11].

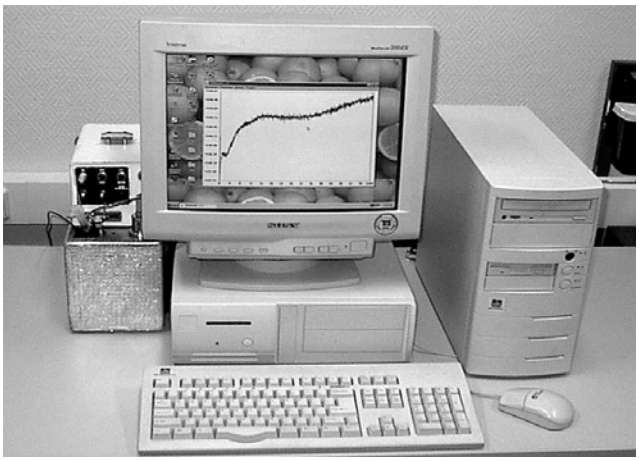

Fig. 1. **Ultrasonic whole blood coagulometer**

The new whole blood coagulometer was developed in Ultrasound institute (Fig.1). Its operation is based on experimentally established fact that ultrasound velocity in a blood sample is changing in a specific way during blood clotting process. Data of measurements are stored and processed by PC. Sensitivity of measurements is ± 3 cm/s.

The calibration and verification of the whole blood ultrasonic coagulometer, monitoring a whole clotting process from the very beginning (≈ 30 s) to the lysis (≈ 1h) is problematic from the point of view of dynamic process and the lack of existing standards. Requirements for such a reference liquid, mimicking clotting blood are following [12]:

- evolution of ultrasound velocities in the time domain must be close to a most common time dependence of ultrasound velocity in clotting blood;
- changes of ultrasound velocity must be caused by changing physical-chemical properties of the liquid, but not by changes of the temperature;
- acoustic properties of the reference liquid must be reproducible with a high accuracy not only in terms of absolute ultrasound velocity values, but from the point of view or their evolution in the time domain as well.

We have proposed [12, 13] a non-stationary reference medium in which polymerisation reaction takes place for dynamic calibration and periodic checking of the measurement system.

The aim of this investigation is a search of polymerisation compositions with various ultrasound velocity dependencies and their statistical definition with the purpose to fulfil the stated requirements.

Measurement method

Measurement of an ultrasound velocity in a small 0.2 ml volume cell is based on the difference of delay times of the ultrasonic pulses reflected from the buffer rod and the cell bottom. The ultrasound velocity value in the blood sample is expressed as:

$$\hat{c}_{\mathrm{B}}\left[t_i, T(t_i)\right] = \frac{2L_{\mathrm{B}}}{\hat{\tau}_{\mathrm{B}}\left[t_I, T(t_i)\right]}, \qquad (1)$$

where $\hat{c}_{\mathrm{B}}\left[t_i, T(t_i)\right]$ is the ultrasound velocity in the sample and i is the measurement number, T is temperature of the sample, L_{B} is the length of measurement cell, $\hat{\tau}_{\mathrm{B}}\left[t_i, T(t_i)\right]$ is the measurement difference of the time intervals.

The influence of temperature on the ultrasound velocity is eliminated providing experiment at a stable 37ºC temperature within ± 0.01ºC, so velocity variations reflect only structural changes of the blood sample.

Polymerisation reaction was investigated in the same dismountable for cleaning purposes cell (Fig.2) at the same temperature as blood clotting was monitored.

Fig. 2. **The measurement cell**

Experimental

Acrylamide CH_2=$CHCONH_2$ is a white water-soluble crystalline solid. Physical properties of acrylamide are the following: molecular weight is 71.08, melting point is 84.5ºC, density at 30ºC is 1.1222 g/mole, solubility in water at 30ºC is 215.5 g/100 ml. Acrylamide was supplied by the Company Sigma-Aldrich Chemia GmbH, Germany, 99+ % electrophoresis grade (79-06-1).

Free-radical chain polymerisation is a chain reaction consisting of three sequential steps: initiations, propagation, and termination. The polymerisation reaction begins with the initiation step, which is the formation of free radicals. Potassium peroxodisulphate $K_2S_2O_8$ as a simple free radical initiator was used at first. The Company Lachema, Czech Republic, supplied $K_2S_2O_8$. The thermal decomposition of persulfate yields two species capable of initiating polymerisation: sulfate radical-ion and the hydroxyl radical:

$$S_2O_8^{-2} \xrightarrow{k_1} 2\,SO_4^{-1}\cdot, \qquad (2a)$$

$$SO_4^{-1}\cdot + HOH \xrightarrow{k_2} HSO_4^{-1} + \cdot OH. \qquad (2b)$$

The rate at which this initiation step occurs is described by equation; $-d[C]/dt = k_d[C]$, where $[C]$ is the concentration of the initiator and k_d is the rate constant for radical dissociation. Following the formation of free radical, the second part of the initiation step involves the addition of these free radicals to monomer molecules. It is known that the hydroxyl radical is poor initiator of polymerisation [14]. So, the formation of a polymer chain via radical addition to the acrylamide is illustrated in equation:

$$CH_2{=}CH + KSO_4\cdot \xrightarrow{k_{i1}} KSO_4 - CH_2 - HC\bullet \quad (CONH_2;\ CONH_2), \qquad (3)$$

i.e. radicals react with a monomer witch lead to an adduct new radical on the carbon. Polymerisation consists of the successive addition of monomer molecules to the polymer chain. It is assumed that the initiation step is very fast compared to radical formation. Therefore $-d[C]/dt = k_d[C]$ is assumed to be the rate limiting step in initiation. Upon initiation, the chain then grows via propagation, as shown in equation (4):

$$KSO_4{-}CH_2{-}HC\bullet + CH_2{=}CH \xrightarrow{k_p} KSO_4{-}CH_2{-}CH_2{-}HC\bullet \quad (CONH_2;\ CONH_2;\ CONH_2). \qquad (4)$$

In general the propagation step can be represented as follows:

$$M_n\cdot + M_1 \xrightarrow{k_p} M_{n+1}. \qquad (5)$$

The kinetic rate expression for propagation is assumed to be independent of chain length. Therefore, all chains terminated by a free radical have the same reactivity. The rate of monomer disappearance (i.e., rate of polymerisation) is the sum of the rates of initiation and propagation:

$$-d[M]/dt = R_I + R_p. \qquad (6)$$

The rate of initiation is negligible compared to the rate of propagation. Therefore, the rate of propagation is the sum of many individual propagation steps:

$$-d[M]/dt = k_p[M][M^*], \qquad (7)$$

$[M]$ is the monomer concentration, $[M^*]$ is the concentration of chain ending free radicals, and k_p is the rate constant for propagation.

The termination step can take by several mechanisms. First, two growing chains can combine and form a carbon-carbon bond, effectively killing both chains. This is known as coupling. Second, disproportion occurs, when one growing chain abstracts a radical from another growing chain causing termination of one by a simple CH_3 group, and the other by a double bond. In either termination leads to the disappearance of two radicals. The rate of termination can be expressed as:

$$R_t = 2k_t[M^*], \qquad (8)$$

where k_t is the rate constant for termination.

Ultrasonic wave propagation is sensitive to growing of polymer macromolecular chains and intermolecular interactions. Fig.3 presents the course of polymerisation

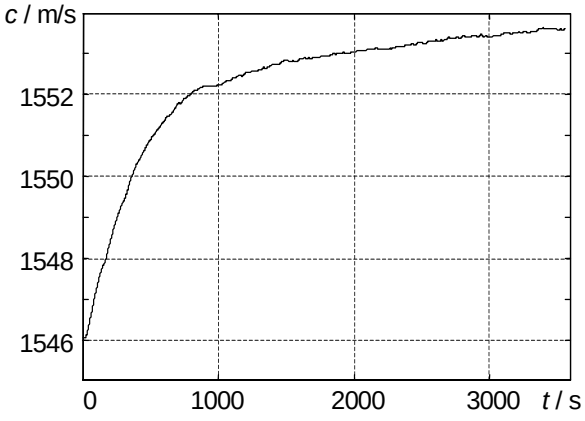

Fig.3. **Course of acrylamide polymerisation initiated by potassium peroxodisulphate**

reaction of acrylamide at initial monomer concentration 7% on mixture weigh and at concentration of $K_2S_2O_8$ 0.04 moles/litre.

As seen, during the polymerisation ultrasound velocity monotonically grows up to the termination of the reaction. The curve doesn′t reflected the ability of coagulometer to react to the rather fast ultrasound velocity changes observed in the beginning of coagulation (Fig.4).

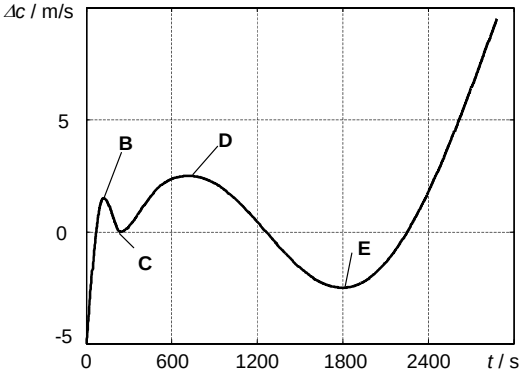

Fig.4. **Generalized blood coagulation curve. The "zero" point in the Δc axis is equal to the value of ultrasound velocity at the point C of the curve**

The curve in Fig. 4 present peculiar stages of blood coagulation of healthy person characterised by their duration and intensities, i.e. ultrasound velocity changes. Point A signs the moment of the injection of the blood into the cell, and the temperature stabilisation of the sample takes place in the time interval AB (till ~ 20-30 s). Points B and D mark the beginning and the end of blood clotting, at point C ultrasound velocity minimum is observed frequently. Interval DE represents so called latent refraction after which main retraction and lysis take place [6]. The most informative is blood clotting process B-C-D The whole process is characterised by a complicated dependence of ultrasound velocity in time.

Thus, the persulfate-thiosulfate redox couple has been investigated also as an initiation system in the polymerisation of acrylamide. Thiosulfate is acted as accelerator of polymerisation reaction. The amount of thiosulfate must be less than 0.001 % of monomer weigh in order to active radicals not be transformed to ions. Sodium thiosulphate pentahydrate $Na_2S_2O_3{\times}5H_2O$ was supplied by the Company Lachema, Czech Republic.

The stoichiometry or persulfate-thiosulfate redox couple is following by the reaction:

$$S_2O_8^{-2} + 2\ S_2O_3^{-2} \rightarrow S_2O_6^{-2} + 2\ SO_4^{-2} \qquad (9)$$

Fig.5 shows two various courses of polymerization with time when thiosulfate concentrations are the same ($1.5{\times}10^{-4}$ moles/litre), but it presents in different quantities: 5a – 0.25 ml, 5b – 0.15 ml. Fig.6 presents the polymerisation reaction curve what fits the best the blood clotting process. Here thiosulfate concentration and amount is same more less (Tables 1 and 2). The effect of thiosulfate on the character and rate of polymerisation is evident. Due to the lower quantity of thiosulfate initial peak at 20-30 s increases from signs only in Fig.5a to the ~0.5 m/s in Fig.6. Intensity of polymerisation and its rate in the interval CD fall gradually: 2.0→1.5→1.3 m/s and 2.5→2.1→1.6 cm/s in 10 s accordingly.

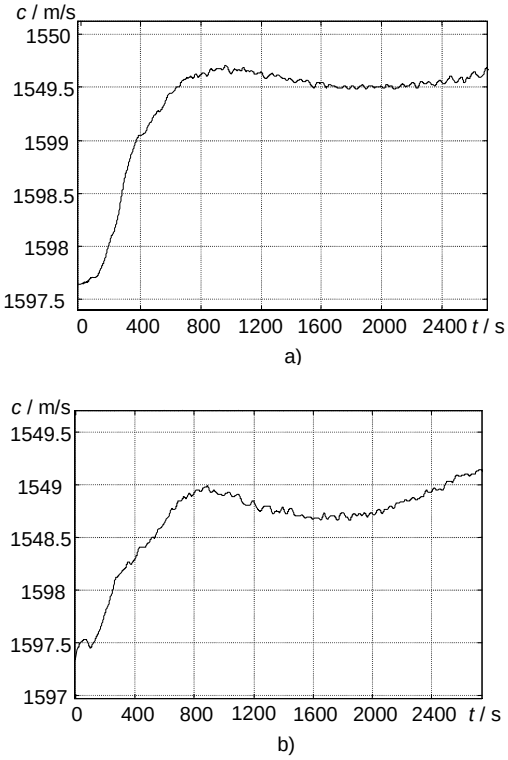

Fig.5. **Acrylamide polymerisation initiated by the persufate-thiosulfate redox couple at different quantities of thiosulfate: a-0.25 ml, b- 0.15 ml**

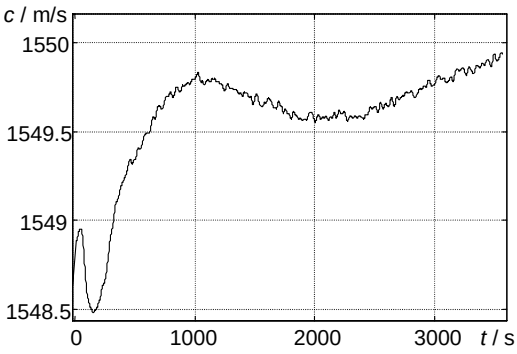

Fig.6. **Optimal polymerisation reaction**

In a general case ultrasound velocity depends on a molecular weight and density. As seen, polymerisation reaction demonstrates a wide range of effects on molecular weight dependence resulting from the generation of different initiator species. The length of chain reaction depends on the ratio of amount of active particles that are generated in time unit to numbers of these particles, which are produced for growing radical. At the interval BC the ultrasound velocity decreases rapidly to a minimum; it is assumed that thermal decomposition of persulfate is negligible, and that $k[S_2O_3^{-}\cdot] >> k[SO_4^{-}\cdot]$. Increasing the thiosulfate concentration accelerates reactions:

$$S_2O_8^{-2} + S_2O_3^{-2} \xrightarrow{k_1} SO_4^{-1}\cdot + SO_4^{-2} + S_2O_3^{-1} \qquad (10)$$

and

$$M_f + S_2O_3^{-2} \xrightarrow{k_{dt}} P_fS + SO_3^{-2} \quad (11)$$

proportionately. From point C ultrasound velocity increases also rapidly and thereafter at interval DE changes monotonically. The consumption of thiosulfate in reactions:

$$S_2O_3^{-2} + CH_2{=}CHCONH_2 + HOH \xrightarrow{k_5} {}^{-1}S_2O_3CH_2CH_2CONH_2 + OH^{-1} \quad (12)$$

and

$$SO_4{\times}S_2O_3^{-2} + S_2O_3^{-2} \xrightarrow{Fast} S_4O_6^{-2} + SO_4^{-2} \quad (13)$$

increases the rate by competing with reaction (11), and the reaction with hydroxyl radical in reaction:

$$\cdot OH + S_2O_3^{-2} \xrightarrow{k_6} S_2O_3^{-1}\cdot + OH^{-1} \quad (14)$$

decreases the concentration of initiating species thus decreasing the rate of reaction:

$$\cdot OH + M_1 \xrightarrow{k_{i2}} M_1 \quad (15)$$

Optimal composition of acrylamide compound mixture is presented in the Table 1.

Table 1. **Composition of acrylamide polymerisation compound mixture**

Materials	Formula	Concentration
Acrylamide	$CH_2{=}CHNH_2$	7 % on mixture weigh
Potassium persulphate	$K_2S_2O_8$	0.04 moles/litre
Sodium thiosulphate pentahydrate	$Na_2S_2O_3{\times}5H_2O$	$1{\times}10^{-4}$ moles/litre.
Distilled water	H_2O	Laboratory grade

Twenty experiments were carried out with the same composition. The solution of monomer was prepared newly before each experiment, while solutions of inorganic salts could be used up to 4 days. Errors of the initial solutions preparation and the amounts of sample are given the Table 2. In order to achieve better accuracy of experiment appropriate quantities of materials were dissolved in large volumes of water. Sodium thiosulphate solution concentration and amount is characterised with the worst accuracy.

Data of mean velocities $\hat{c}$, times $\hat{t}$ and standard deviations σ_c, σ_{cn}, σ_t in characteristic points for twenty experiments with optimal composition are presented in Table 3. Standard deviation σ_{cn} is σ_c normalised according to the ultrasound velocity value at the point C. At the point A an ultrasound velocity depends on the injection procedures, initial compound room temperature as well, not only on compound composition.

The mean acrylamide polymerisation curve averaged from 20 separate experiments is shown in Fig.7. It represents clearly the main phases, typical for whole blood coagulation process and shows the ability of coagulometer to register fast ultrasound velocity variations.

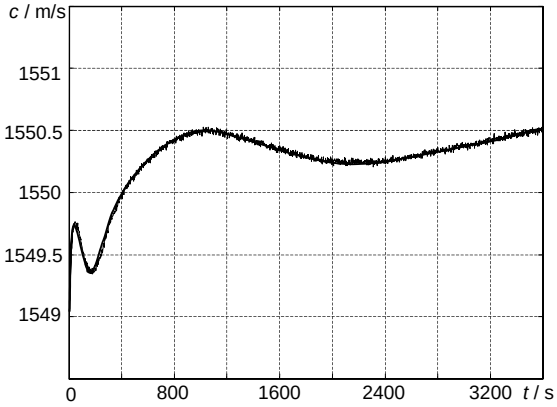

Fig.7. **The mean acrylamide polymerisation curve for optimal composition averaged from 20 experiments**

Table 2**. Errors of the solution's preparation**

Materials	Balance	Weight		Water volume		Total solution error	Amount	
		g	error	ml	error		ml	error
Acrylamide	Electronic	3.5	±0.3 %	50.0	±0.1 %	±0.32 %	5.0	±0.5 %
Potassium persulphate	Analytic	0.5406	±0.02 %	50.0	±0.1 %	±0.1 %	1.4	±0.35 %
Sodium thiosulphate	Torsion	0.0062	±1.6 %	250	±0.04 %	±1.6 %	0.14	±3.6 %
Distilled water							3.5	±0.7 %

Table 3. **Mean values of ultrasound velocity and standard deviations at characteristic points for 20 experiments**

Parameters	Points				
	A	B	C	D	E
$\hat{c}$, m/s	1548.27	1549.07	1548.63	1549.86	1549.52
$\hat{t}$, s	0.00	24.7	78.4	507.8	1141
σ_c, m/s	0.82	0.90	0.84	0.79	0.88
σ_{cn}, m/s	0.32	0.15	0.00	0.34	0.36
σ_t, s	0.0	7.6	13.7	73.7	144

Standard deviation of the ultrasound velocity σ_c, and the standard deviation σ_{cn}, normalised to the ultrasound velocity at the point C for the measurement data are shown in Fig.8 and Fig.9 accordingly.

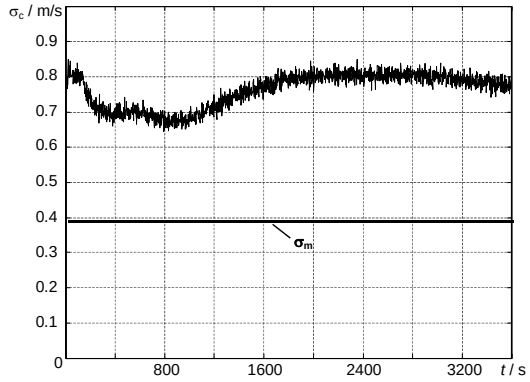

Fig. 8. **Standard deviation of the ultrasound velocity σ_c for the whole polymerisation curve. σ_m – standard deviation of the measurement results caused by disassembling and assembling of the measurement cell for cleaning purposes**

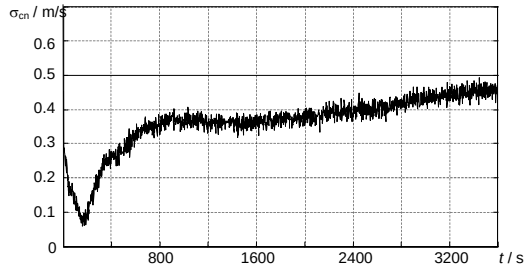

Fig.9. **Normalized standard deviation σ_{cn},**

Conclusions

Free-radical chain polymerisation reaction of acrylamide initiated by a certain ratio of persulphate and thiosulphate is characterised by fast and slow variations of ultrasound velocity in the time domain in different reaction phases, which is close to observed in clotting blood. Twenty experiments were carried out with the optimal composition of acrylamide compound mixture. Errors of solutions preparation and ultrasound velocity variation identity are presented. This chemical polymerisation reaction of acrylamide can be used for a dynamic calibration of the ultrasonic coagulometer.

Acknowledgement

The support provided to this research by the Lithuanian National Science and Studies Foundation is gratefully acknowledged.

References

1. International workshop on the standardization of whole blood coagulation devices. Whole blood coagulation workshop meeting (8/13/99). http://www.fda.gov/cdrh/meetings/coag.html.
2. Sonoclot coagulation & platelet function analyzer with graphics printer. For *in vitro* diagnostic use. http://www.uscid.org/~sieco/operatorsmanuals.html.
3. **Jacobs J., E., Malinka A., V., Haque P., Jhabvala M.** Ultrasound spectroscopy applied to blood coagulation studies. Ultrasonics. Elsevier. 1975. Vol. 3. P.84.
4. **Voleišis A., Kažys R., Mažeika L., Šliteris R., Voleišienė B.** Ultrasonic technique for the investigation of structural properties of biological fluids. *In*: 137th Meeting of the Acoustical Society of America and the 2nd Convention of the European Acoustics Association, Forum Acusticum, Berlin, Collected papers, CD-ROM, Technishe Universitaet. 1999. P. 4.
5. **Grybauskas P.** Detection of blood coagulation disturbances in ischemic heart disease by means of new ultrasound coagulometer. *In*: XIIth Meeting Int. Soc. Hematol., Vienna. 1993. Abstr. 345. P. 87.
6. **Grybauskas P.** In: Ultrasonic Coagulometry. Kaunas. Medical Academy. Kaunas. 1998. P. 281 (in Lithuanian).
7. **Voleišis A., Kažys R., Mažeika L., Šliteris R., Voleišienė B.** Method of investigation of non-stationary biological fluids. Ultragarsas (Ultrasound). Kaunas: Technologija. 1999. No.1(31). P.35-39.
8. **Feng R.** Investigation of ultrasonic properties of blood. In: Proc. China-Japan. Joint. Conf. Ultrasonic. 1987. P.11.
9. **Funk T., Eggers F.** Clotting of blood at a gold surface probed by MHz shear quartz resonator. Naturwissenschaften. 1982. Vol.69(2). P.499.
10. **Machado J. C., Von Kruger M. A., Foutes E. M A., Almeida M.M.G.** Evaluation of an ultrasonic method applied to the measurement of blood coagulation time. Physiol. Meas. 1997. Vol.18. P.129.
11. Medical CT and ultrasound: current technology and applications. Publishers "American association of physicists in medicine". 1995.
12. **Voleišis A., Kažys R., Mažeika L., Šliteris R., Voleišienė B., Grybauskas P.** Ultrasonic method for the whole blood coagulation analysis. Ultrasonics, Elsevier. 2002. Vol.40. P. 101-107.
13. **Voleišienė B., Mongirdienė A., Voleišis A., Šliteris R.** Reference medium for blood coagulation ultrasonic studies. Ultragarsas (Ultrasound). Kaunas:Technologija. 2000. No.1(34). P. 20-22.
14. **Riggs I.P., Rodriguez F.** Persulfate-initiated polymerization of acrylamide. Journ. Polymer Science. 1967. Pt.A-1. 5. P.3151-3165.

B. Voleišienė, A. Voleišis, R. Kažys, L. Mažeika, R. Šliteris

Dinaminis kalibravimas ultragarsinėje koagulometrijoje

Reziumė

Ištirta radikalinė akrilamido polimerizacijos reakcija, inicijuota kalio persulfato, taip pat kalio persulfato ir natrio tiosulfato poros. Parodyta, kad esant tam tikroms reagentų proporcijoms ultragarso greičių kitimų pobūdis polimerizacijos metu yra toks pat kaip ir krešant kraujui. Pateikiama optimalios polimerizacijos reakcijos matavimų rezultatų analizė. Apskaičiuotos tirpalų paruošimo ir matavimų paklaidos. Akrilamido polimerizacijos reakciją siūloma taikyti ultragarsinio kraujo koagulometro dinaminiam kalibravimui.

Pateikta spaudai 2002 11 5