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New flow-through analytical system based on ion-selective field effect transistors with optimised calcium selective photocurable membrane for bovine serum analysis

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ABSTRACT

An analytical system based on ion-selective field effect transistors (ISFETs) with Ca^{2+} ion selective photocurable membranes is developed to offer a semiautomatic analysis of serum calcium concentration of ruminants. Optimisation of ion-sensitive membrane composition containing different copolymerised plasticizers was performed to minimise the effect of a highly lipophilic samples on sensors characteristics. To improve the system reliability the set of calibration solutions and measurement sequence were also optimised. The system was used to determine total calcium concentration in bovine sera. The precision of the ion determination was higher than reported for double charge ions with a standard deviation of about 3–7%. It is shown that the presence of coagulant in blood serum samples does not affect the determined total calcium concentration.

1. Introduction

The history of the cow's milk fever study began in the 1920s when analysis of ionic compounds of cow's serum sample confirmed the hypothesis that this disease provokes decrease of calcium concentration level for these animals [1]. Milk fever, which is a severe form of hypocalcemia, in dairy cows is an economically important disease and significantly increases a cow's susceptibility to mastitis, retained foetal membranes, displaced abomasum, dystocia and ketosis, which can reduce a cow's productive life [2]. Currently, producers can readily identify animals with milk fever through the observation of overt clinical signs, including dull appearance, lethargy, cold ears, or a down cow [3]. In this connexion, the development of portable, ease to use, semiautomatic measuring system for serum calcium concentration might be very beneficial for small and middle farms, because 47% of all cows in their second lactation or greater had varying degrees of subclinical hypocalcemia that in some cases is severe enough to alter physiological and immune functions [4].

Traditionally, assessment of an animal's calcium status is based on evaluation of the total calcium concentration (tCa). The tCa concentration is assumed to be directly proportional to ionized calcium (iCa), which is the biologically active form of calcium and is regarded as the gold standard for determination of calcium status [5]. Normal serum calcium concentrations in healthy mid-lactation cows range from 2.1 to 2.8 mmol L^{-1} and from 1.6 to 2.6 mmol L^{-1} during the week after calving among cows with no subsequent clinical disease [6]. Subclinical hypocalcemia can be more difficult to diagnose, because it is characterised by low serum calcium concentration in the absence of clinical milk fever symptoms. Values of $\leq$1.8 mmol L^{-1} for serum calcium in the first week after calving have been proposed as a suitable threshold for the diagnosis of subclinical hypocalcemia [7].

Previously we have reported about application of an analytical system based on optimised flow-through cell and ion selective field effect transistors (ISFETs) with sensitive membranes for blood serum ion analysis [8]. Application of photocurable polymers and the photolithography technique for ISFET encapsulation, as well as for ion-sensitive membrane formation [9] showed the feasibility of this approach that helps to make the process of ISFET fabrication more technologically favourable. To enhance the performance and the lifetime of cation sensitive polymer membranes a copolymerisable plasticizer di-(n-hexyl)-itaconate has been used. The developed system allows to determine ion concentration of sodium, potassium, chloride ions and pH in blood serum samples with a standard deviation of 3–5% [8].

The novelty of the present work lies in optimisation of the composition of previously proposed [10] calcium ion selective photocurable polyurethane membranes in terms of their stability and selectivity and application of the optimised Ca-ISFETs within a

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semiautomatic flow-through measuring system for analysis of serum calcium concentration of animals in cattle farms.

2. Experimental

2.1. Reagents and solutions

Commercial calcium Ionophore II, plasticizer bis(2-ethylhexyl) sebacate (DOS), lipophilic additives potassium tetrakis(p-chlorophenyl)borate (K-TpClPB) and ETH 500, as well as hexafluorobutyl acrylate HFBuA were purchased from Fluka. Copolymerisable plasticizers di-(n-hexyl)-itaconate (DHI) was synthesised in the laboratory [8]. Two other copolymerisable plasticzers, di-(n-octyl)-itaconate (DOI) and di-(methylheptyl)-itaconate (DMHI), were synthesised according to procedure presented below. Aliphatic urethane diacrylate (oligomer Ebecryl 270) and cross-linker, hexanediol diacrylate (HDDA), were from UCB Chemicals. Photoinitiators 2,2′-dymetoxyphenylacetophenone (IRG 651) and 2-hydroxy-2-methyl-1-phenyl-propane-1-one (DAR 1173) were from Ciba-Geigy. All other chemicals used were of analytical-reagent grade. Standard solutions were prepared with deionised water.

2.2. Synthesis of copolimerisable plasticizers di-(n-octyl)-itaconate (DOI) and di-(methylheptyl)-itaconate (DMHI).

2.2.1. Di-(n-octyl)-itaconate

Place a mixture of 39 g (0.3 M) of itaconic acid, 78 g (0.6 M) of 1-Octyl alcohol, 300 ml of sodium-dried toluene and 1 g of p-toluenesulfonic acid in a 1-litre round-bottomed flask fitted with Dean-Stark trap. Reflux the mixture, with stirring, for 6–8 h or until water no longer collects in appreciable amount in the water separator, then allow to cool. Pour the reaction mixture into excess of water (2–3 volumes), separate the toluene layer, wash it with saturated sodium bicarbonate solution until effervescence ceases, then with water, and dry with anhydrous sodium sulphate. Remove most of the toluene by distillation under normal pressure until the temperature rises to 120 °C, then distil under reduced pressure and collect the di-(n-octyl)-itaconate at 190–195 °C/0.1 mm. The yield of the above reaction was 53 g (52%). The obtained product was characterised by ^{1}H NMR spectroscopy giving the following spectrum: ^{1}H NMR (300 MHz, DMSO): 6.20 (s, 1H); 5.71 (s, 1H); 4.16–3.97 (m, 4H); 3.27 (s, 2H); 1.64–1.58 (m, 4H); 1.38 (m, 20H); 0.90 (m, 6H). Elemental data (calculated for $C_{21}H_{38}O_4$) was: 69.8 (71.1)% C, 10.6 (10.8)% H.

2.2.2. Di-(methylhepyl)-itaconate

In the same manner was obtained di-(methylhepyl)-itaconate from 0.15 M of itaconic acid and 0.3 M of 2-octyl alcohol. T_b=185–187 °C/0.1 mm. The yield is 20 g (45%). Spectrum data ^{1}H NMR (300 MHz, DMSO): 6.17 (s, 1H); 5.67 (s, 1H); 4.78–4.87 (m, 2H); 3.23 (s, 2H); 1.56–1.46 (m, 4H); 1.16–1.27 (m, 22H); and 0.88 (m, 6H).

Elemental data (calculated for $C_{21}H_{38}O_4$) was: 70.8 (71.1)% C, 10.9 (10.8)% H.

2.3. Preparation of ion-selective membranes

Photocurable membrane composition was prepared as presented elsewhere [10]. First the main polymer composition was prepared by mixing together the aliphatic urethane diacrylate oligomer Ebecryl 270, reactive diluent HDDA and photoinitiator Irgacure 651 in an 81:17:2 w/w ratio. Only in the case of membrane composition MCa1 (see Table 1) the main polymer composition was as reported earlier [8] and consisted of Ebecryl 270, DHI and photoinitiator DAR 1173 in a 59:36:5 w/w ratio.

Table 1
Studied Ca^{2+}-selective ISFETs membrane compositions, required exposition time and selectivity over sodium ions.

Membrane	Ionophore ETH 129, (wt%)	Lipophilic additives, (mol%)	Plasticizer, (wt%)	Exposition time, (s)	$-\log K_{Ca/Na}$
MCa0	1.0	K-TpClPB, 56	DOS, 40	15	3.5
MCa1[a]	1.8	K-TpClPB, 56 ETH 500, 21	DHI, 36	200	Anionic influence
MCa2[a]	1.7	K-TpClPB, 58	DHI, 36	60	1.3
MCa3	1.0	K-TpClPB, 58	DHI, 41	200	2.1
MCa4	1.8	K-TpClPB, 71	DHI, 37	200	1.7
MCa5	1.1	K-TpClPB, 53	DOS, 20 DHI, 20	90	1st day—3.4 15–2.4 29–1.9 43–1.6 164–0.8
MCa6	1.0	K-TpClPB, 57	DOS, 10 DHI, 30	60	1st day—3.4 15–2.5 29–2.3 111–1.1 164–1.0
MCa7	1.0	K-TpClPB, 53	DOI, 39	60	0.6
MCa8	1.0	K-TpClPB, 53	DMHI, 40	75	0.4
MCa9	0.9	K-TpClPB, 56	HFBuA, 36	60	High resistance
MCa10	1.0	K-TpClPB, 54	HFBuA, 38	20	0
MCa11	1.0	K-TpClPB, 57 ETH 500, 20	DOS, 20 HFBuA, 20	20	1st day—3.6 8–3.6 27–3.6 86–3.3

[a] DAR 1173 was used as a photoinitiator.

In the next step 0.3 g of the main polymer composition was dissolved in 0.2 ml of tetrahydrofuran and to this solution plasticizer if needed (35–40% w/w), ionophore and lipophilic salts were added. The mixture was thoroughly stirred in an ultrasonic bath until homogeneous and then left for several hours to evaporate the solvent. Compositions of studied calcium selective membranes are given in Table 1.

2.4. Sensor fabrication

Sensors were made using n-channel ISFETs with SiO_2 gate insulator. After scribing and wire bonding ISFETs were encapsulated with photocurable polymer composition, as presented elsewhere [11]. To enhance the adhesion of the acrylated urethane polymer ISFET devices were preliminarily silylated by exposure to a 10% (v/v) (methacryloxy)propyltrimethoxysilane solution in methanol with a subsequent heat treatment during 1 h under 80 °C in an oven. The membrane composition was then delivered by a microsyringe into the well formed by encapsulated layer over the gate region of an ISFET and was exposed to UV using UV Curing Light Lamp System PC-5000 (Dymax) with irradiance of 62 mW cm^{-2} at the wavelength of 365 nm. Exact values of exposure time are presented in Table 1. This resulted in a polymer membrane formation with the thickness of 150–200 μm.

2.5. Evaluation of chemical response

Initially, for optimisation of membrane compositions individual ISFET sensors were used. At least 4 devices were studied to determine the properties of photocurable membranes. These experiments were performed in batch under stationary conditions by addition of prepared stock solutions of $CaCl_2$ in the concentration range from 10^{-7} to 10^{-2} M. Selectivity coefficients for sodium, as the most interfering ion in serum analysis, were determined by the mixed solution method on the background of 0.1 M concentration of interfering ion.

ISFET devices have been measured in a conventional manner in a constant drain current mode (I_D=100 μA, V_D=0.5 V) using a computer controlled experimental set-up. A double-junction Ag/AgCl reference electrode (Orion 90-02) was used as an external electrode with a 0.1 M solution of lithium acetate as a salt bridge.

2.6. Flow-through system

The designed automatic system is shown schematically in Fig. 1. To pump solutions and deliver them to the flow cells (4) a syringe pump (1) is used, while a multiposition valve (3) permits to select among various solutions (calibration solutions or samples) and to collect them in the holding coil (2) before injecting into the flow cells. The high precision syringe pump used operates in the flow rate range of 1–500 μL sec^{-1} and with a sample volume of 1–1000 μL with a ±0.1 μL precision.

A flow cell that incorporates two sensors and the reference electrode was developed and fabricated from a polymethylmethacrylate (PMMA) plate by micromilling (Stepfour GMbH, Salzburg, Austria). Different milled parts of the PMMA were glued together with methacrylic acid. The inner fluidic channels were 0.5 mm high and 0.8 mm wide with the total length of 50 mm. The external dimension of the cell was 12 × 81 × 21 mm^3. Flow cell contains two separate cameras for each sensor (3 mm in diameter and 0.5 mm high). Standard 0.8 mm inner diameter tubing for flow injection analysis was connected to the inlet and outlet by ¼"-28 UNF connectors. For tight contact between the flow cell and sensors polydimethylsiloxane gasket was used and the cell was fixed by screws.

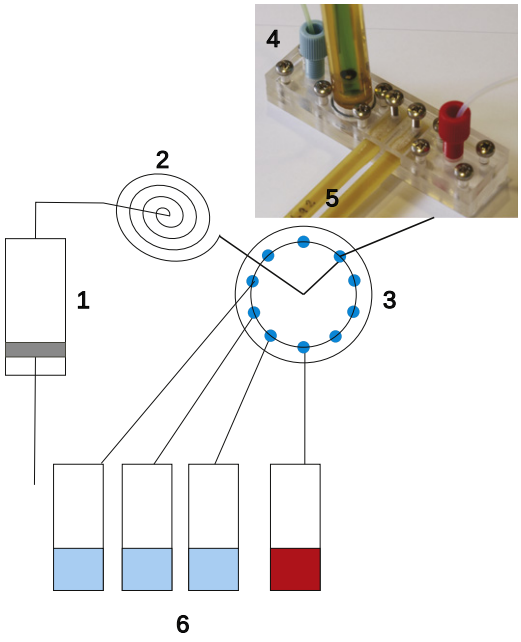

Fig. 1. The designed automatic flow-through system. 1—syringe pump, 2—holding coil, 3—multi-position valve, 4—flow cell, 5—ISFET sensors, and 6—calibration and test solutions.

Calibration of the system under the flow rate of 20 μL sec^{-1} and solutions volume of 1000 μL lasts about 5–7 min and automatic analysis for each unknown sample takes about 2 min.

2.7. Recollection of serum samples

Thirteen healthy Holstein lactating cows from IRTA experimental farm (Monells, Spain) were used. Blood samples of the tail vein from each cow were collected in Vacutainer tubes (ref. BD367896) without additives or in tubes containing thrombin (ref. BDV368923, Beckton Dickinson, Rutherford, NJ, USA). From thrombin tubes serum was directly recovered after coagulation. Serum from blood samples without additives were recovered after centrifugation at 2000 × *g* for 15 min. Serum samples were stored at −20 °C until further analysis.

2.8. Blood serum measurements in a flow-through system

For the determination of total calcium in blood serum the procedure published by Anker et al. [12] was applied. Calibration solutions contained 1, 2 and 9 mM Ca^{2+} in the presence of 140 mM Na^+, 4.5 mM K^+ and 0.8 mM Mg^{2+}. The serum samples as well as calibration solutions were diluted with a 20-fold volume of acetate buffer with pH 3.5. This dilution of the sample permits to convert all forms of calcium (protein-bound calcium and calcium complexes with other possible ligands) into the ionized form. The buffer was prepared by mixing 0.382 M of acetic acid, 0.02 M of NaOH and deionised water in a 1000 ml flask. The slope of the sensors response was determined by measuring in 1 and 9 mM Ca^{2+} diluted solutions before and after serum sample measurements. The 2 mM Ca^{2+} diluted solution was measured in alternation with a blood serum sample also diluted with a buffer. The total calcium concentration was determined from the potential difference between the 2 mM Ca^{2+} calibration solution and serum sample taking into account the actual slope of the ISFET sensor.

2.9. Inductively coupled plasma optical emission spectrometry (ICP-OES)

As an independent method of total calcium determination inductively coupled plasma optical emission spectrometry (ICP-OES) was used. 100 μL of serum was diluted with 2 mL of solution containing 0.05% EDTA in 0.5% NH_4OH. The measurements were carried out on ICP-OES spectrometer (Perkin-Elmer, model Optima 4300DV) in the department of Chemical Analysis of Autonomous University of Barcelona (UAB).

3. Results and discussion

3.1. Optimisation of Ca-selective photocurable membrane

Initial experiments performed under flow conditions showed that ion selective membranes prepared with DOS as a plasticizer as reported earlier [10] (MCa0, Table 1) have limited lifetime and within a week lost their mechanical solidity due to more rapid, compared to stationary conditions, leaching of the plasticizer. Typically in Ca-selective membranes oNPOE or 4-nitrophenyl phenyl ether (NPPE) is used for better selectivity. Unfortunately this type of membrane solvents cannot be used in photocurable membranes because nitro group inhibits the radical initiation of the polymerization. Attempts to exclude the plasticizer from the membrane composition and variation of the polymer/cross-linker (aliphatic urethane diacrylate/hexanediol diacrylate) ratio were

not successful due to high electrical resistance of the resulting membranes.

Taking into consideration that earlier studied K^+ and Na^+ selective membranes with copolymerisable plasticizer di-(n-hexyl)-itaconate [8] showed promising results, the first membrane composition with this plasticizer (MCa1, Table 1) was prepared using the same main polymer composition (Ebecryl 270, DHI and photoinitiator DAR 1173 in 59:36:5 w/w ratio) together with lipophilic additives and the ionophore. Unfortunately, obtained calibration curve was nonlinear and starting from 5×10^{-4} M of $CaCl_2$ strong anionic influence was observed. Also for this composition (MCa1) the required polymer curing time was longer (200 s) than in the case of normal plasticizer like DOS (see Table 1). This prolonged UV exposure results in photobleaching and decomposition of K-TpClPB [13] which affects the selectivity of membranes, as the selectivity of potentiometric sensors for double charge ions highly depends on a molar relation between the ionophore and lipophilic additives [14,15]. To reduce the curing time 9% of HDDA (w/w) was added to the membrane composition (MCa2) and although this permits to decrease the curing time from 200 s to 60 and obtain linear calibration curve in a 10^{-5} to 10^{-1} M concentration interval, the selectivity of this membrane was insufficient (Table 1).

Assuming that the loss of selectivity over sodium from 3.5 (MCa0) to 1.3 for MCa2 may be related with another type of used photoinitiator (DAR 1173) and/or with the products of photochemical reactions, we returned to initial composition MCa0 using copolymerisable plasticizer DHI instead of DOS (composition MCa3). This substitution provokes increase in membrane curing time till 200 s. The selectivity was better than in the case of MCa2 composition, but not sufficient for serum analysis. Increase in the ionophore/lipophilic additive molar ratio up to 71 mol% of K-TpClPB (MCa4) did not improve the selectivity.

It must be noted that the absence of free plasticizer within the membrane matrix may cause two problems. Firstly, the solubility of an ionophore and lipophilic additives in the membrane mixture will be decreased, which may seriously affect the sensor parameters like sensitivity, selectivity and the limit of detection. Secondly, this will affect the mobility of charged species within the membrane phase which will result in a very high membrane impedance (50–200 MΩ) [13]. As we reported earlier, copolymerisable plasticizer DHI may successfully used for sodium, potassium and pH ion-selective membranes [8,16], however in the case of Ca^{2+} ion selective membranes the results were not favourable. It is possible to enhance the selectivity over sodium ions by using the mixture of two plasticizer DOS and DHI in 1:1 and 2:1 w/w ratio (membrane compositions MCa5 and MCa6, respectively), but starting from the second week of the constant contact with water solutions selectivity coefficient deteriorate (see Table 1).

An improvement of the selectivity coefficient may be achieved by increasing the length of the alkyl chains of plasticizer [17]. For this di-(n-octyl)-itaconate and di-(methylhepyl)-itaconate were synthesised and introduced in membrane compositions MCa7 and MCa8, respectively, but unfortunately the desired effect was not achieved and selectivity of sensors with these membranes was very low.

Another way to alter the selectivity of polymer ion-selective membranes is by changing the nature of polar moieties in the polymer structure. For example, by introduction of cyano, nitroether, amide, keto or alkylester groups in polydimethylsiloxane membranes sensors with good response to nitrate ions were obtained [18]. However, the synthesis of these siloxane monomers and polymers seem to require too much efforts. The sensors selective to K^+ and Ca^{2+} with good analytical performance were obtained by UV copolymerisation of commercially available oligodimethylsiloxane methacrylate with comonomers carrying polar moieties [19]. So, in the next step of our investigations we introduced one of comonomers studied in this work, hexafluorobutyl acrylate (HFBuA). This introduction of 36% (w/w) of HFBuA as a copolymerisable plasticizer (MCa9) resulted in membrane with very high resistance (more than 10 MΩ) that provoked the oscillation of ISFET-meter circuitry [13]. To reduced membrane resistance we eliminated the crosslinker HDDA from the main polymer composition (MCa10) which allowed us to obtain calibration curve of the sensor but selectivity of these membranes was very low. Taking into account the behaviour of membranes MCa5 and MCa6, the mixture (1:1) of HFBuA and DOS was used for membrane composition MCa11. In this case sensors with Nernstian sensitivity of 28.3 ± 0.3 mV/pCa, linear response in the 0.1 M to 10^{-5} M concentration range and the detection limit of 3×10^{-6} M were obtained. Sensors showed very good selectivity and stable parameters of calibration curves that permitted us to apply them for the determination of total calcium in bovine blood serum.

Starting from the paper of Fogt et al. [20] published in 1985, it is universally [21] assumed that polymer membrane ISFETs with a membrane deposited directly over a silicon oxide or silicon nitride gate inevitably suffer from the interference caused by penetration of the carbon dioxide and organic acids from a water solution to the membrane organic phase. In the case of photocurable membranes as we have shown [22] that the presence of CO_2 and acetate ions in test solutions have no effect on ISFET sensors response. This means that no water layer is formed at the membrane/dielectric interface where acidification, affecting an ISFET signal, may occur due to penetration of CO_2 or acetic acid. This may be attributed to technological peculiarities of the sensor design [11], when the membrane composition is deposited into a well formed by a polymer encapsulating layer which prevents possible lateral attack of water at the membrane/insulator interface. It also should be mentioned that adhesion of photocured polyurethane membranes to the silylated surface of silicon dioxide is very good.

The influence of the solution pH changes on calcium selective ISFETs response was tested in a 5×10^{-2} M TRIS buffer containing 5×10^{-3} M $CaCl_2$. The solution pH was changed by adding drops of a solution containing 1 M HCl and 5×10^{-3} M $CaCl_2$. Results show that studied polyurethane membrane ISFETs do not have any pH response in the 2.0–5.5 pH range and small pH sensitivity of 2–3 mV/pH between 5.5 and 9.3 values. These results coincided with previous data obtained for calcium selective membrane with DOS as a plasticizer [10]. The absence of pH influence in the 2.0–5.5 pH range permits calibration of the sensors and sample measurements in the presence of acetate buffer (see Section 2) with good precision. The sensors response slope obtained with calibration solutions of 1, 2 and 9 mM Ca^{2+} in the presence of 140 mM Na^+, 4.5 mM K^+ and 0.8 mM Mg^{2+} was 28.3 ± 0.3 mV/pCa. The same solutions diluted with 20-fold volume of acetate buffer yield a response with the slope of 28.8 ± 0.4 mV/pCa.

3.2. Analysis of serum samples

Typical sequences of calibration solutions and serum samples used in the total calcium measurement cycle in a flow through cell with two Ca^{2+}-ISFET sensors are shown in Fig. 2. Obtained results along with total calcium concentration determined by optical emission spectrometry (ICP-OES) are given in Table 2.

Each serum sample listed in Table 2 was measured by potentiometric method three times and, as can be seen from the presented results, the precision of total calcium determination is very high. Typical accuracy of a potentiometric method for double charge ions concentration measurement according to the Nernst equation is 8–12% and depends on the stability of a sensor potential and junction potential of the reference electrode [23]. Comparing the results obtained by the potentiometric method and

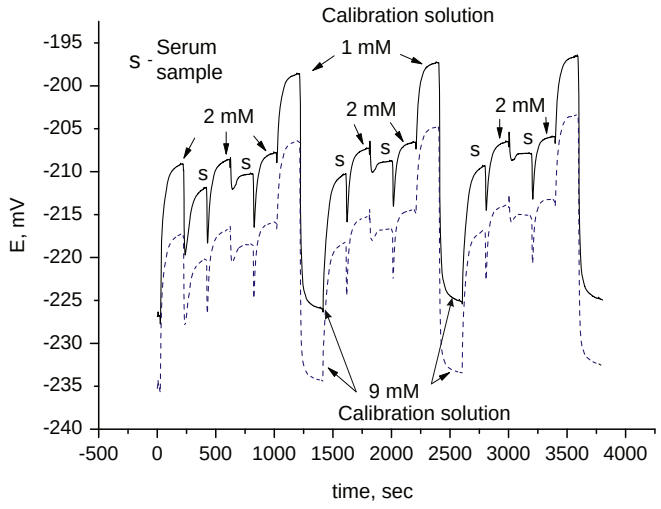

Fig. 2. Response of two sensors (solid and dashed line) to a typical sequence of calibration solutions and serum samples.

Table 2
Total calcium concentration (mmol L^{-1}) in bovine sera determined by potentiometric (ISFETs) and inductively coupled plasma optical emission spectrometry (ICP-OES) methods.

Sample	ISFETs	ICP-OES[a]
10	2.4 ± 0.2	2.3
18	2.2 ± 0.1	2.1
26	2.1 ± 0.1	2.2
41	2.2 ± 0.1	2.1
89	2.1 ± 0.1	2.2
96	2.5 ± 0.1	2.4
100	2.2 ± 0.1	2.3
136	2.4 ± 0.1	2.3
560	2.2 ± 0.1	2.3
561	2.1 ± 0.1	2.1

[a] Precision of the OES method is 0.8–1.4%.

ICP-OES we may conclude that the accuracy of calcium measurements with ISFET sensors is within 3–7%, which shows really high selectivity and stability of the developed ion selective membranes and the flow-through system as a whole.

Table 3 presents results of the total calcium concentration determined in the flow-through system in serum samples obtained by centrifugation and by coagulation in thrombin test-tubes. As follows from the presented results there are no differences between calcium total concentrations in samples with added coagulant and without it. This means that in the case of potential application of the developed measuring system in cattle farms it will be possible to avoid the process of centrifugation of blood samples and to obtain serum samples by coagulation, which is more economic and easier method.

4. Conclusion

In this work application of the ISFET sensors with membrane sensitive to calcium ions for analysis of bovine serum is reported. To enhance the performance and the lifetime of calcium ion sensitive polymer membranes different types of copolymerisable plasticizers were synthesised and investigated. Optimised membrane composition contains a mixture of free (dioctyl sebacate, DOS) and copolymerisable (hexafluorobutyl acrylate, HFBuA) plasticizers, shows Nernstian response and sufficient selectivity over sodium, potassium and magnesium ions present in their physiological concentrations. Analytical system based on sequential injection analysis was developed and optimised in terms of the flow conditions, measurement sequence, and calibration solutions composition. The developed system permits to determine ion concentration of total calcium in blood serum samples with standard deviation of 3-7% and may be easily adapted as a handheld instrument for field measurements directly at a farm.

Table 3
Total calcium concentration (mmol L^{-1}) determined in serum samples obtained by centrifugation and by coagulation in thrombin test-tubes.

Serum sample	Centrifugation	Thrombin
134	2.0 ± 0.1	1.9 ± 0.1
138	1.8 ± 0.1	1.8 ± 0.1
142	1.9 ± 0.1	1.9 ± 0.1

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