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Influence of O_2- and H_2O_2-Detection on the Electroanalytic Reliability of L-Lactate- and ß-D-Glucose-Biosensors at Flow-Through Measurements in Reference Serum

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Abstract. Electroanalytical flow-through measurements with L-lactate- and ß-D-glucose-biosensors using reference sera showed that only by an ion-impermeable but gas-permeable PTFE membrane separating enzyme membrane and electrochemical measuring cell, the high selectivity of oxidases can fully be utilized. For clinical-chemical serum analysis biosensors with O_2-detectors are to be preferred which are at least equivalent to photometric comparing methods. Too high values found in measurements using H_2O_2-detectors are caused by undesired reactands being anodically converted. At O_2-sensitive enzymatic L-lactate- and ß-D-glucose-biosensors, inevitable biological fouling by O_2-consuming microorganisms can be controlled by the use of glutardialdehyde.

The first description of enzyme electrodes for glucose measurements basing on pO_2- or pH-electrodes using glucose-oxidase membranes was published by Clark and Lyons in 1962 [1]. In the meantime, plenty of reports on L-lactate [2–9] and ß-D-glucose [2, 7, 9–23] measurements with multiple variations of the enzyme membranes have appeared, presently favouring H_2O_2-detection [4–6, 9, 15, 21–23] or the redoxmediator-rendered electron transport between enzyme and platinum anode [2, 7, 8, 10, 11, 20]. Immobilization of enzymes by adsorption [24] basing on hydrogen junctions, interactions of charge and hydrophobe regions or insertion into polyacrylamide matrix [16] allows the biocatalysts to be washed out [25] of the membrane systems. In contrast to the macroincapsulation of linked enzyme molecules between two microbially impermeable dialysis membranes [26] we favour, the frequently employed covalent linkage of enzyme molecules to bovine albumin [6, 17, 19] and other matrices such as collagen [13, 23], PVA [10] or PVC [12], which yields little resistance to protein catabolism by bacteria or fungi. Formerly unrecognized and uncontrollable biological fouling [26] is one of the reasons to prefer H_2O_2- to O_2-detecting sensors in bioelectrochemical analyses of L-lactate and ß-D-glucose with substratum high-selective oxidases, in spite of well known interferences regarding anodically reacting substances such as uric acid, ascorbic acid [7], and also acetaminophen-type pharmaca. Protein layers being adsorbed to channel sides of electroanalytical flow-through measuring systems provide a perfect nutrient medium for microorganisms with consecutive O_2-consumption and hereby falsification of the measuring signal [26].

By use of glutardialdehyde (GDA) biological fouling on O_2-detecting enzymatic L-lactate- and ß-D-glucose-sensors can be suppressed [26], so that the selectivity raising effect of ion-impermeable but gas-permeable polytetrafluorethylen(PTFE)-foil separating enzyme membrane and platinum cathode can reliably be utilized.

Experimental

Using molecular selective oxidases and measuring either O_2-consumption or H_2O_2-production, the electrochemical-enzymatic analysis of L-lactate and ß-D-glucose takes place according to the following ways of reaction :

$$\text{L-lactate} + O_2 \xrightarrow{\text{LOD}} H_2O_2 + \text{pyruvate}$$

$$\text{ß-D-glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} H_2O_2 + \text{gluconic acid}$$

Through cross-linkage, 40 U of either L-lactate-oxidase (LOD) from *Pediococcus species* (SBC-1212, SBC-1222) or ß-D-glucose-oxidase from *Aspergillus niger* (SBC-1010) were fixed between two sterile and microbially impermeable dia-lysis membranes using glutardialdehyde. Immobilization of LOD resp. GOD by covalent linkage guarantees reliable functioning of the enzyme membranes in continuous flow-through usage. Forming a barrier to non-volatile molecules, the ion-impermeable PTFE-membrane of the O_2-sensor constitutes an excellent contribution to selectivity improvement of the gas sensitive detectors. The membrane systems of biosensors integrated into flow-through systems form the top of the reservoir-shaped flow-through chamber with channels for access and outlet facing each other.

Fig.1 shows in principle the general construction of molecular selective biosensors with cathodic and anodic method of detection. An explicit discussion on both bioelectrochemical measuring principles has been given in [26].

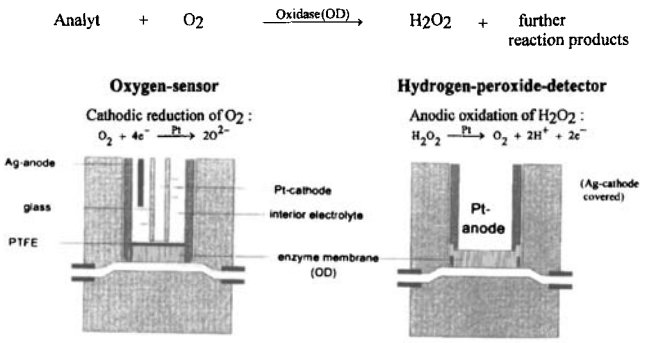

Fig. 1 Functioning principles of L-lactate- and ß-D-glucose-sensors with O_2- and H_2O_2-detectors.

While the plateau-adjusted polarisation voltage of oxygen-sensors was 750 mV, the hydrogen peroxide detector must have a polarisation voltage of 950 mV when no environmentally straining redox mediators such as benzochinone [2] are used. At increasing analyt concentration, O_2-sensitive enzymatic biosensors show lower, H_2O_2-detecting sensors show higher measuring current. The calculation of each analyt concentration can be done according to the subsequent equation [26], which, because of the functions' contrary slope, is given in two variations :

Molecular selective sensor with O_2-detector :

$$c_x = c_1 + \frac{I_1 - I_x}{I_1 - I_2} \cdot (c_2 - c_1)$$

Molecular selective sensor with H_2O_2-detector :

$$c_x = c_1 + \frac{I_x - I_1}{I_2 - I_1} \cdot (c_2 - c_1)$$

- c_x concentration of the analyt in the measuring solution
- c_1 lower concentration of substratum in the calibration solution K_1
- c_2 higher concentration of substratum in the calibration solution K_2
- I_x current intensity according to c_x in the measuring solution
- I_1 current intensity according to c_1 in K_1
- I_2 current intensity according to c_2 in K_2

By suction of a roller pump the measuring solution flows through the sensor. For the intermediate-carrier analysis system with rapid dialysis [26, 27], an oxygenator pump is integrated between dialysis chamber and the pressure-adjusting chamber preceding the sensor [26]. The samples are separated by air bubbles.

For fouling-resistent functioning, O_2-sensitive enzymatic biosensors in continuous flow-through usage demand the application of bactericide glutardialdehyde. With a concentration of 0.01% in the carrier solution, GDA is permanently present in the carrier current and is compatible to the enzyme membrane [26]. In case of uncontrolled exacerbation of microbial growth, additionally 1 ml of 2.5 % glutardialdehyde

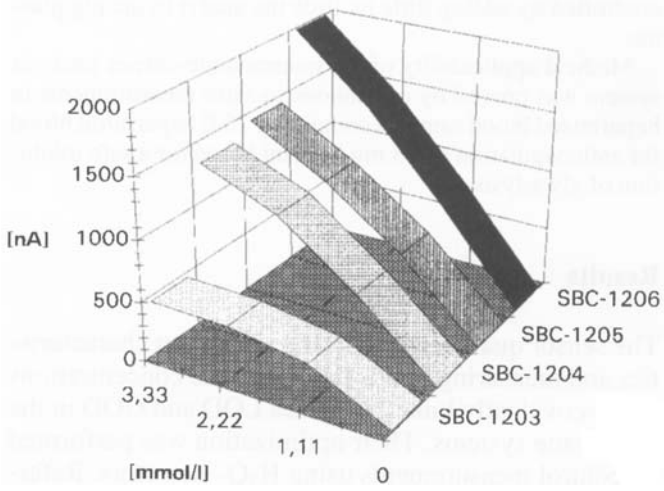

Fig. 2 Coherence of LOD-concentration in the enzyme membrane and linearity of measuring range.

Membrane code	Enzyme concentration
SBC-1203	5 U/Membrane
SBC-1204	15 U/Membrane
SBC-1205	20 U/Membrane
SBC-1206	35 U/Membrane

solution is given through the dialysator. For direct measurement, microbial spreading in the flow-through system channels is suppressed by application of 1 ml 0.25 % glutardialdehyde solution once every 24 hours.

The reference sera Qualitrol® N, Qualitrol® L and Qualitrol® H from the E. Merck company (Darmstadt) of animal origin were used. Pig's blood served for plasma analyses of L-lactate and ß-D-glucose. Instant prevention of coagulation and glycolysis was guaranteed by adding 200 ml of 2% sodium citrate solution plus 3 mg NaF/ml sample to 500 ml of the blood specimen. The plasma was then produced by centrifugation as usual. For direct measurements, plasma specimen and reference sera were previously diluted 1:21 by aqueous phosphate-buffered solution (PPL) of pH = 7.04.

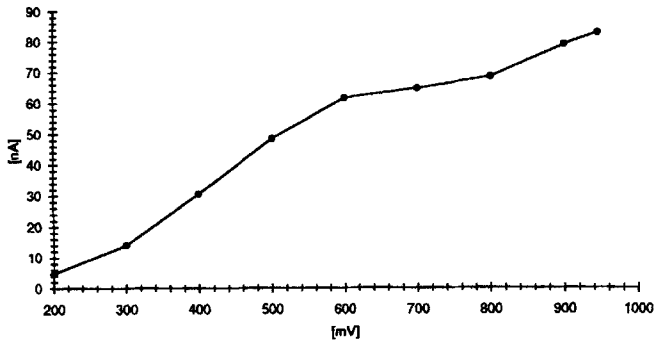

Fig. 3a Polarogram of a L-lactate biosensor with O_2-detector at continuous flow-through measurement of L-lactate-free phosphate-buffer solution equilibrated by air oxygen at 20° C and 1017 hPa.

The linearity throughout the whole measuring range was controlled by adding little by little the analyt to the pig plasma.

Medical applicability of the intermediate-carrier analysis system was proved by continuous in-vitro measurements in heparinised blood samples containing 15 E heparin/ml blood for anticoagulation and 3 mg NaF/ml blood for a safe inhibition of glycolysis.

Results

The sensor qualities concerning resolution characteristics and measuring range depend on the concentrations of the covalently linked enzymes LOD and GOD in the membrane systems. Their optimization was performed by control measurements using H_2O_2-detectors. Referring to O_2-consuming oxidases, the maximum of enzyme activity inserted into the membrane system has to be adjusted to the oxygen content of the measuring medium (Fig. 2).

Setting up a polarogram for the O_2-detecting sensor can be done by using the oxygen given in the measuring solution (Fig. 3a), whereas the H_2O_2-detector apart from the enzyme membrane also requires the relating substratum in order to set up a polarogram by continuous production of hydrogen peroxide. Regarding this fact we chose for the L-lactate sensor a concentration of 5.0 mg/dl (0.56 mmol/l) (Fig. 3b) and for the ß-D-glucose-sensor of 100.0 mg/dl (5.55 mmol/l) of the analyt. For better estimation of the adequate polarisation voltage for the two H_2O_2-detecting sensors, a three-dimensional polarogram by variation of both polarisation voltage and concentration of L-lactate resp. ß-D-glucose is necessary (Fig. 3c). Referring to Fig. 3b and 3c the polarisation voltage required for operation of L-lactate- and ß-D-glucose sensors has to be 950 mV. Fur-

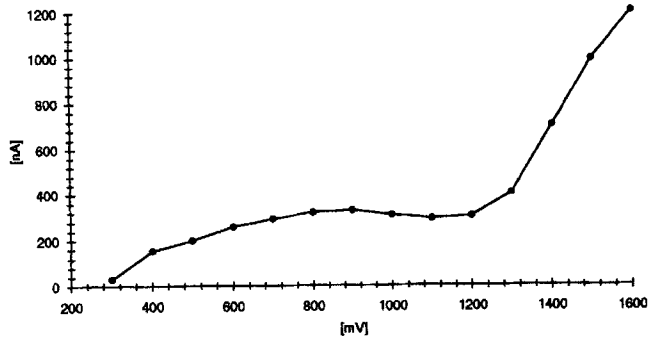

Fig. 3b Polarogram of a L-lactate biosensor with H_2O_2-detector at continuous flow-through measurement of 0.56 mM L-lactate phosphate-buffer solution equilibrated by air oxygen at 20° C and 1021 hPa.

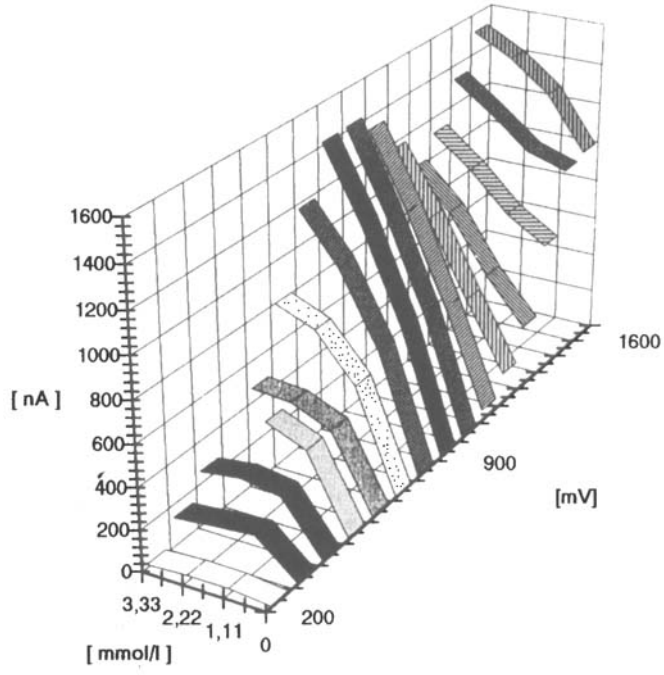

Fig. 3c Three-dimensional polarogram according to the conditions given in Fig. 3b under variation of L-lactate concentration from 1.11 mmol/l to 3.33 mmol/l.

ther raising of the polarisation voltage lessens the electrode's functioning time, also changing its mode of response. It is well known that for lower polarisation voltage redoxmediators are indispensable for the electron transport between oxidase and the amperometric redox electrode of platinum.

At the ß-D-glucose sensor, direct anodic glucose oxidation leads to an electrochemical measuring current superposing the enzyme reaction and intensifying the signal. At the L-lactate-sensor, however, this means an unwanted interference (Fig. 4a).

The direct anodic turnover of ß-D-glucose showed a maximum at freshly polished platinum electrodes, then sank drastically according to an e-function within a period of two weeks and could finally no longer be traced (Fig. 4b). This implies not clearly calculable time-dependent glucose interferences at L-lactate-sensors with H_2O_2-detection. Other undesired anodic electrode processes are caused, for example, by urea acid, ascorbinic acid and acetaminophen.

This kind of detector-dependent interference does not occur at O_2-electrodes, since the ion-impermeable but gas-permeable PTFE-membrane between enzyme membrane and platinum cathode serves as electrically insulating diffusion barrier and can well be utilized for highly selective serum analyses. So far, permanent routine application of O_2-sensitive enzymatic L-lactate- and ß-D-glucose sensors in protein containing measuring solutions was hindered by an uncontrollable biological fouling, which, up to now had not been recognized. This fundamental problem was solved by using glutardialdehyde [26].

For serum and plasma analyses, O_2-consuming enzyme reactions as well as O_2-detectors require constant conditions concerning pO_2. These conditions and the constant pH-value with regard to the enzyme reactions are guaranteed by 1:21 dilution with a Na/K-phosphate-buffer at pH = 7.04 (PPL).

On this basis, a three point calibration was carried out with 30.0, 50.0 and 100.0 mg/dl (3.33, 5.55 and 11.10 mmol/l) L-lactate in aqueous phosphate-buffered solution (PPL) for the measuring range of the L-lactate sensor from 0.1–100.0 mg/dl (0.01–11.10 mmol/l). Separated by air bubbles, the sample to be analysed is transported into the sensor after having supplied the medi-

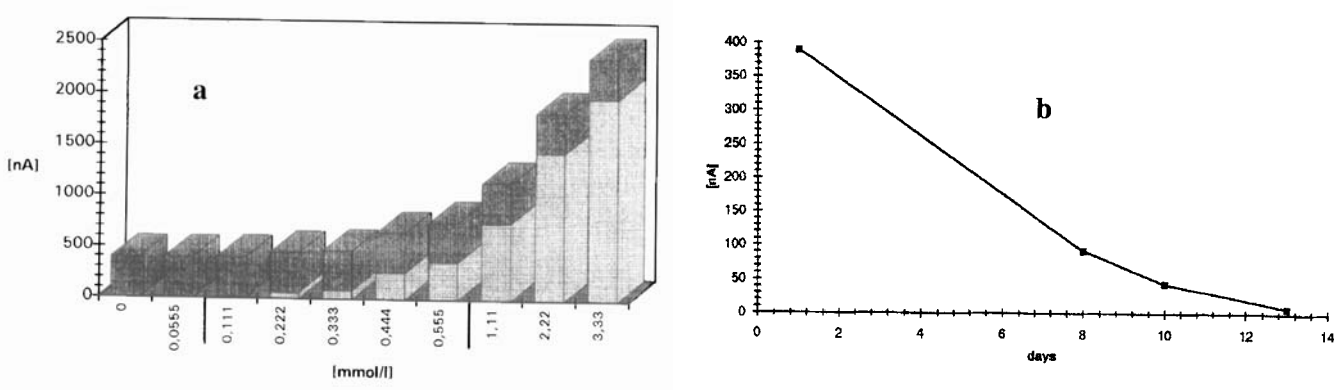

Fig. 4 Glucose (5.55 mmol/l) interference of L-lactate sensors with H_2O_2-detection
a) with freshly polished platinum anode b) e-functional decrease of interference current within two weeks.

um calibration solution. Depending on the current measured at the specimen, the second calibration solution is then chosen at 30.0 mg/dl (3.33 mmol/l) or 100.0 mg/dl (11.10 mmol/l) so that a two-point calibration is given with the specimen's concentration in between. In conformance to the calibration of the L-lactate sensor calibration points for the ß-D-glucose-sensor were given at 100.0, 400.0 and 800.0 mg/dl (5.55, 22.20 and 44.41 mmol/l). For very low concentrations of the analyt in the specimen, a forth calibration point may be necessary. In analyses by intermediate-carrier system, a linear measuring range is found for ß-D-glucose concentrations up to 1000.0 mg/dl (55.51 mmol).

In clinical-chemical analyses of L-lactate and ß-D-glucose, the addition of sodium fluoride for glycolysis prevention is a well known technique. A concentration of 6 mg/ml NaF in undiluted aqueous solutions, however, leads to a reversible inhibition of the covalently linked LOD. For glycolysis prevention, 2 mg NaF/ml blood are supposed to be sufficient [28].

For direct blood measurements, the dialysis chamber's diluting effect (1:11) is sufficient to reduce the NaF concentration in front of the sensor membrane to an extent where it does not inhibit the enzyme activity. Therefore, no measurable influences of the glycolysis inhibitor on the activity of the enzyme membranes were found when measuring the plasma without dialysator

Table 1 Quantitative biosensor characteristics (cp. Fig. 5)

L-lactate sensor :			**ß-D-glucose sensor:**		
day A (measurements: 1 - 10)			**day A** (measurements: 1 - 10)		
	O_2-detector	**H_2O_2-detector**		**O_2-detector**	**H_2O_2-detector**
Mean value	5.49 mmol/l	6.06 mmol/l	Mean value	5.68 mmol/l	6.15 mmol/l
Standard deviation	0.10 mmol/l	0.05 mmol/l	Standard deviation	0.08 mmol/l	0.07 mmol/l
Variation coefficient:	1.82%	0.83%	Variation coefficient	1.41%	1.14 %
day B (measurements: 11 - 20)			**day B** (measurements: 11 - 20)		
	O_2-detector	**H_2O_2-detector**		**O_2-detector**	**H_2O_2-detector**
Mean value	5.52 mmol/l	6.05 mmol/l	Mean value	5.48 mmol/l	6.16 mmol/l
Standard deviation	0.08 mmol/l	0.07 mmol/l	Standard deviation	0.03 mmol/l	0.03 mmol/l
Variation coefficient	1.45%	1.16%	Variation coefficient	0.55 %	0.49 %

by using a 1:21 dilution with PPL.

Repeatability (precision in series) and reproducibility (day-to-day precision) were examined by 10 measurements in flow-through technique on two succeeding days, using reference serum Qualitrol® N and L-lactate- and ß-D-glucose-biosensors for H_2O_2- and O_2-detectors with identical enzyme membranes. The results found concerning mean value, standard deviation and variation coefficient are presented in table 1. As to the electrochemical-analytical reliability of L-lactate- and ß-D-glucose-sensors in dependence of the two detection methods, the graphical presentations of Fig. 5 prove that only O_2-sensitive enzymatic measurements stay within the demanded target range of the reference serum Qualitrol® N, which is a very reliable reference method. The reliability of O_2-sensitive enzymatic methods is supported by measurements in reference serum Qualitrol® H for L-lactate (Fig. 6, below), and ß-D-glucose (Table 2), and also in Qualitrol® L for L-lactate (Fig. 6, above). The deviation of ß-D-glucose as found

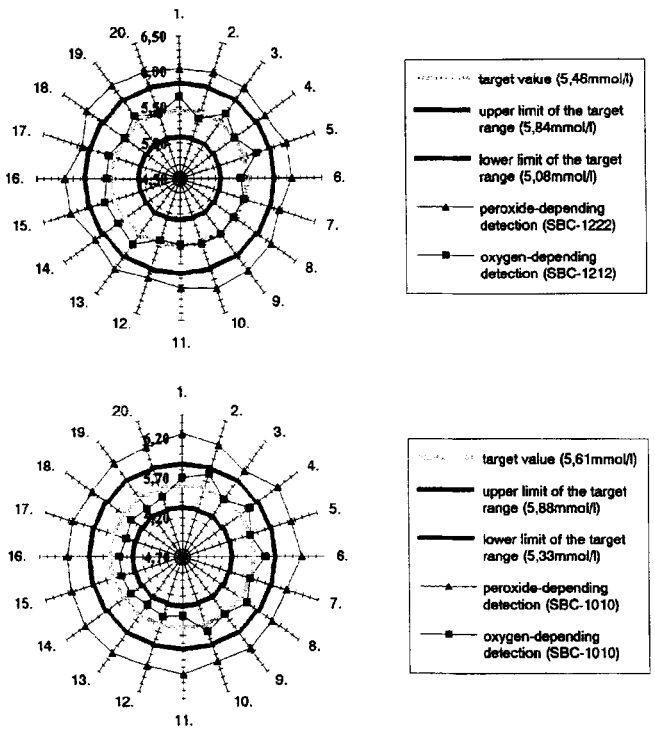

Fig. 5 Influence of O_2- and H_2O_2-detection of L-lactate- and ß-D-glucose-biosensors in flow-through measurements with reference serum Qualitrol® N in reticulated diagraphic presentation (cp. table 1).

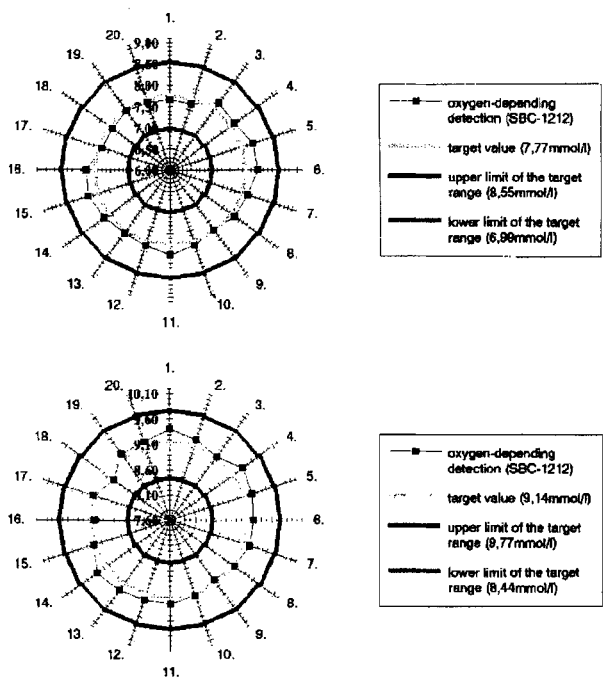

Fig. 6 Reticulated diagrams for L-lactate biosensors with O_2-detector at flow-through measurements in the reference sera Qualitrol® L (above) and Qualitrol® H (below) on two succeeding days with 10 analyses each day.

O_2-sensitive enzymatically in Qualitrol® L (table 2) is twice as high if the H_2O_2-detecting method is used (not presented).

There is a remarkable shift of the target ranges between the different photometric analyses methods for the determination of glucose, with simultaneous divergence for the different reference sera Qualitrol® N, L and H (Tab. 3).

Table 2 Amperometric ß-D-glucose-sensor with O2-detector (SBC 1010). Electroanalytical measurements in reference sera diluted 1:21 by PPL.Reference sera: Q_N: Qualitrol® N; Q_H: Qualitrol® H; Q_L: Qualitrol® L
Precision in series (repeatability) and day-to-day precision (reproducibility).
Pt-diameter : 400 µm , U_{pol}: 750mV ; Setting time of the sensor : 1-2 minutes. Values and standard deviation (St. Dev.) in mmol/l; Variation coefficient (Var. Co.) in %

	day A			day B		
No.	Q_N	Q_H	Q_L	Q_N	Q_H	Q_L
1	5.70	11.14	2.83	5.45	11.17	2.83
2	5.80	11.04	2.78	5.50	11.09	2.83
3	5.60	11.14	2.78	5.45	11.19	2.88
4	5.75	11.04	2.78	5.50	11.14	2.88
6	5.75	11.02	2.78	5.50	11.14	2.88
7	5.60	11.10	2.83	5.45	11.22	2.88
8	5.70	11.05	2.78	5.50	11.07	2.88
9	5.60	11.14	2.83	5.45	11.24	2.87
10	5.70	11.04	2.78	5.50	11.14	2.81
Mean	5.68	11.08	2.80	5.48	11.16	2.86
St. Dev.	0.08	0.05	0.03	0.03	0.06	0.03
Var. Co.	1.41	0.45	1.07	0.55	0.54	1.05

Discussion

Unknown biological fouling was up to now a hindrance for the application of reliable and stable O_2-sensitive enzymatic measuring systems [26]. Since flow-through channels and membrane surfaces of bioelectrochemical measuring systems cannot be kept sterile on the long run, the forming of biological films on adsorbed protein layers predestinates the settlement of O_2-consuming aerobic microbes, being increased by the presence of adequate nutrition. The supply of oxygen as reactant for the enzyme reaction in the biosensor membrane is already reduced by the omnipresence of biological layers on the tubular system sides. In addition to the mentioned O_2-consumption [26], chemical changes by influence on pH and ionic strength [29] of the measuring medium are observed. Physical effects appear as increase of friction [29] and change of surface qualities of the flow-through system sides. The unwelcome higher adherence of air bubbles is a sign for lower moistening. The change of redox potentials at metal surfaces leads to electrochemical fouling at H_2O_2-detectors.

The solution of the problem was found by using glutardialdehyde, thus suppressing the O_2-consumption of aerobic microbes in the biological coating of the whole flow-through system including the sensor membranes. A failure of the effect of glutardialdehyde regarding development of resistance could not be observed within one year's use of GDA in our laboratories.

Table 3 Reference sera : Glucose concentration according to the declarations of the producer. The values obtained from ß-D-glucose-biosensors within GOD-membranes must be compared with the photometric GOD-method.). All values in mmol/l

Method of analysis	Reference sera:	**Qualitrol N**	**Qualitrol H**	**Qualitrol- L**
Reference method	Target value	5.12	11.00	2.33
	Target range	–	–	–
	Recommended value	4.35–5.89	9.4–12.7	1.99–2.66
Gluc-DH	Target value	5.34	10.9	2.28
	Target range	5.08–5.61	9.9–11.8	2.05–2.50
	Recommended value	4.54–6.15	9.2–12.5	1.94–2.61
GOD-PAP	Target value	5.61	10.9	2.33
	Target range	5.33–5.89	10.0–11.9	2.10–2.55
	Recommended value	4.76–6.45	9.3–12.5	1.99 –2.66
Hexokinase	Target value	5.26	11.4	2.28
	Target range	5.00–5.52	11.0–11.9	2.05–2.50
	Recommended value	4.47–6.05	9.7–13.2	1.94–2.61

Basing on an anytime suppressible microbial growth in biological coating and therefore controllable biological fouling, the reliable applicability of highly selective bioelectrochemical sensors for analyses of L-lactate and ß-D-glucose could be realised.

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