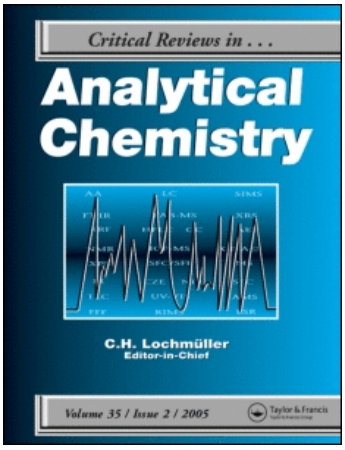

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Current Trends in Enzymatic Determination of Glycerol

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Current Trends in Enzymatic Determination of Glycerol

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This review focuses on the enzymatic glycerol detection methods. Importance of glycerol as biologically active compound, chemical glycerol determination methods, and current trends in enzymatic glycerol determination are reviewed. Application of FAD-dependent glycerol oxidases, NAD-dependent dehydrogenases, lipases and multi-enzymatic systems for glycerol and polyglyceride detection by optical and electrochemical methods are analyzed in detail. The application of PQQ-dependent enzymes is predicted as one of alternative and promising ways for detection of glycerol in complex biological samples. Future trends in application of PQQ-dependent glycerol dehydrogenases are predicted.

Keywords biosensor, PQQ, glycerol dehydrogenase, polypyrrole, electrochemical detection, food, beverage, wine

INTRODUCTION

Recently food and beverage analysis is becoming highly important in analytical chemistry, because the presence/concentrations of metal ions (1) and some organics (2, 3) is playing a role on various biological functions of living organisms (4). Detection of trace metals in wine (1), pesticides and polychlorinated biphenyls in biological samples and foods (2), pesticide residues in juice and beverages (3) were critically reviewed in this journal. According to our opinion presented in this article glycerol possesses physiological activity and its determination in food and beverages is important task of analytical chemistry. However, the detection of this analyte in complex biological samples is still complicated and needs to be improved. To solve this problem alternative ways based on application of biosensors might be applied.

Importance of Glycerol Determination. Glycerol is the backbone of triglycerides, the most important storage form of animal and vegetal fats and oils. It is an important metabolite in energy metabolism, involved in oxidation and synthetic processes. The two sources of triglycerides in serum are dietary intake and synthesis in fatty acids containing tissues (5). Under various physiological or pathological conditions both sources are metabolized by a variety of lipases to form free fatty acids (i.e., non-esterified) and glycerol. The plasma concentration of glycerol, the backbone of triglycerides and the end product of triacylglycerol breakdown, is considered to reflect lipolysis in adipose tissue. Glycerol released into the systemic circulation is utilized mainly by liver also kidney and muscle (6). That's why glycerol measurement is useful in clinical studies to evaluate lipolysis under various conditions. Glycerol concentration is different for the white and black population, for different age

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TABLE 1
Glycerol occurrence

	Glycerol	Reference
Table wines		
White	56–63° mM	10
Red	67–76° mM	10
Port wine	51–66° mM	10
Desert wine	30–166° mM	11
Grape juice		
White	6.66° mM	12
Red	9.85° mM	12
Honey		
Blossom honey	Up to 300° mg/kg	9
Pastilles	1.482° g/100° g	12
Biomedicine	Triglyceride	Reference
Clinical measurements in general	0.3–2.25° mM	7
Blood		
Newborns	0.3–0.4° mM	13
Children	0.8–1° mM	13
Adults (50–60 years old)		
Men	3° mM	13
Women	2.3–2.4° mM	13

and sex individuals (Table 1). An increased level of triglycerides is an indirect coronary disease risk factor (7). The high serum triglycerides are an independent risk factor for atherosclerosis and various forms of hyperlipoproteinaemia (5). Why, the areas where, it is important to estimate the amount of glycerol are related to its relevance in clinical chemistry, medicine, pharmaceutics and production of food and beverages. In biomedicine, measurements of glycerol are useful for the correction of glycerol interference in the measurement of triglycerides in reference materials and in serum from patients with elevated glycerol concentrations. It has diagnostic value in the identification of patients with a deficiency of glycerol kinase (EC 2.7.1.30), an X-linked inborn error of metabolism characterized by hyperglycerolemia and glyceroluria (8).

The second major area where glycerol concentrations play a very important role is determination of quality and authenticity of food and alcoholic beverages. For example, honey spoilage can be estimated by measuring glycerol content. Harvesting honey with a too high water content leads to spoilage by fermentation, resulting in a product with an off-taste, high levels of yeast, glycerol, butanediol and ethanol (9). A limit of 300 mg/kg glycerol has been proposed for blossom honey (Table 1). Honeydew honeys have higher glycerol content and a limit for these honeys has not yet been proposed.

Glycerol is a secondary product in wine fermentation, contributing to the sensory properties (Table 1). Glycerol is a component of high quality wines. The sweet taste of glycerol gives the wine "body." Adulteration by addition of industrial-grade glycerol to the wine is common case, it can be detected by determining typical substances formed during the production process. If the concentration of glycerol is higher e.g., than 6 to 10% of the ethanol concentration, the wine may be adulterated ("improved") by the addition of glycerol. In other alcoholic beverages, such as beer and distilled spirits, glycerol is also added to improve taste. The genuine Scotch whiskeys are differentiated from lower grade material produced by different manufacturing methods, and sold as Whisky by measuring glycerol content. Adulterated whisky samples are prepared by addition of commercial glycerol to a distilled cane spirit since genuine samples do not posses glycerol. The quality of alcoholic beverages produced from sugarcanes or grapes is then related to the glycerol content. Glycerol is not (or only in traces or small amounts) contained in grape juice from healthy grapes (Table 1). A higher content of glycerol in grape juice may indicate the use of spoiled material in production. So glycerol is subjected to strict supervision prescribed by law, the determination of glycerol should be routinely performed in a wide variety of beverage matrices (14). Moreover, glycerol is one of the most important compounds to be monitored in a sugar-fermentation plant in order to improve the quality control of the process (15).

The third area appendant to industrial glycerol uses e.g., humectant, solvent, plasticizer, soap industry, monitoring of the glycerol concentration in lixivia and as a derivative employed in plastics, coatings, explosives (15). Glycerol is used as a moisturizer in the production e.g., of marzipan, paper, tobacco and cosmetics.

Glycerol concentration in different samples can be assayed using various chemical and enzymatic methods.

The aim of the review is to overlook various glycerol detection methods and to discuss current trends in enzymatic detection of this compound.

DISCUSSION

Chemical Determination of Glycerol

One of the simplest chemical methods involve glycerol oxidation to formaldehyde with periodate and then the formaldehyde formed can be quantitatively measured by various formaldehyde detection methods based on specific reactions with: (i) mixture of chromotropic acid and sulfuric acid spectrophotometrically determined at 570 nm (16); (ii) mixture of phenylhydrazine, ferricyanide and hydrochloric acid spectrophotometrically determined at 540 nm (17); (iii) mixture of 3-methyl-2-benzothiazolinone and ferric chloride spectrophotometrically determined at 620 nm, with ferroin determined at 510 nm (17); (iv) ammonium acetate and acetylacetone colorometrically determined at 412 nm, fluorometrically by using 400 nm primary and 485 nm secondary filters (18). Glycerol oxidation to formaldehyde with periodate might be monitored potentiometrically with periodate ion selective electrode (19). A single-line flow injection system including a tubular periodate-selective electrode without inner reference solution is proposed for glycerol determination in distilled spirit, based on oxidation

of this polyol by periodate. With potentiometric detection, problems associated with the transparency of the samples are not relevant and measurements within a wide concentration range, typically within 4 orders of magnitude, can be performed. Therefore, sample lots with high variability in analyte concentration can be assayed without extensive dilutions. However, the determination can be strongly affected by foreign chemical species usually present in distilled spirits, such as oxidized compounds other than glycerol, and by high ethanol content that may affect the poly(vinyl chloride) membrane of the electrode (14). The official standard methods for glycerol determination in wine are based on spectrophotometric detection of the colored product formed after the reaction of formaldehyde with phloroglucinol, the former being produced after glycerol oxidation by periodate (14).

The official standard methods for the determination of free glycerol in soaps, detergents, cosmetics, etc. were based on its oxidation by peroxidates. The formic acid produced by the oxidation reaction could be titrated with standard sodium hydroxide. I. L. Mattos et al. presented a flow-injection amperometric method for glycerol determination in samples relevant to the industrial production of soaps, detergents and such samples where the glycerol electrocatalytic oxidation in an alkaline medium was exploited. A nickel-chromium alloy micro-cylindric electrode was used as the electrochemical detector. The nickel alloys retained the electrochemical characteristics of the base metal and usually are cheaper than noble metals. Nickel electrodes exhibit catalytic activity in the oxidation of a wide variety of organic compounds in alkaline solutions (15). Glycerol is oxidized by Ni(III), usually described as NiOOH, which is formed in the range from 0.40 to 0.5 V vs. Ag/AgCl. Therefore glycerol can be determinated by monitoring the Ni(II) and re-oxidizing this species by applying a suitable potential (15).

The rate of appearance of glycerol in the systemic circulation can be determined from the enrichment of arterial blood glycerol when ^{2}H-, ^{13}C- or ^{14}C-labelled glycerol is infused intravenously. Lipolysis occurs primarily in adipose tissue, (although other tissues contribute), notable muscle. Measurement is based on the difference in the enrichment of glycerol entering and leaving the tissue. Lipolysis is underestimated by the extent to which glycerol released by lipolysis does not enter the systemic circulation, as occurs when lipolysis takes place in the non-hepatic tissue of the splanchnic bed. Glycerol presenting in systemic circulation is mainly utilized in liver, although kidney and muscle are also important users of glycerol. Measurement of glycerol utilization is based on the amount of labeled glycerol utilized by the tissues (6).

Gas chromatography (20, 21), gas chromatography-mass spectrometry (22, 23) and high-performance liquid chromatography (24–30) can also be used for glycerol determination. These determination procedures are less suitable for glycerol analysis, especially considering the complexity of the investigated matrices (14).

All these above mentioned methodologies are usually expensive and require a long time for sample and standard preparation. Some of them have very specific and sometimes limited use and are not suitable for routine analysis as well as process monitoring. Alternatively to all above described methods enzyme based catalytic assays or biosensors might be developed (31). Completed enzymatic systems eliminate the use of caustic reagents, extraction solvents, high-temperature baths and adsorption mixtures (32).

ENZYMATIC GLYCEROL DETERMINATION

Triglyceride Detection using Lipase

Recent triglyceride and glycerol determination are primarily enzymatic. The first step of triglyceride analysis is hydrolysis of triglycerides to glycerol and fatty acids with lipase (lipolysis) (33–36). R, R′ and R″ represent three different alkyl groups:

$$\begin{array}{l} CH_2OCOR \\ | \\ CH_2OCOR' \\ | \\ CH_2OCOR'' \end{array} + 3H_2O \xrightarrow{\text{Lipase}} \begin{array}{l} CH_2OH \\ | \\ CH_2OH \\ | \\ CH_2OH \end{array} + R'COOH + R''COOH \qquad [1]$$

Triglyceride — Glycerol — Fatty acids

The production of fatty acids by means of lipolysis reaction in the specific reaction conditions may result in pH changes due to dissociation of fatty acids. The change in pH is proportional to the concentration of triglyceride in solution. D. G. Pijanovska et al. presented an indirect potentiometric method for determination of triglycerides based on the pH-determination. Here microreactor packed with lipase immobilized onto a glass beads coated with keratin and nitrocellulose sheets, and the pH-sensor of ISFET type was applied (7). The chemical bond of lipase to the surface of glass beads coated with keratin and entrapment within the alginate gel were found to be effective. The systems consisting of microreactors packed with surface immobilized lipase were adopted for three substrates: triacetin, tributyrin and triolein. The highest sensitivity was obtained for tributyrin, while the widest linear range was obtained for triacetin (7). Enzymatic, porous silicon (PS) based potentiometric method for estimation of triglycerides was reported by R. R. K. Reddy (37). Lipase was immobilized on thermally oxidized p-type (100) crystalline silicon. The pH change was detected by electrolyte-oxidized PS—crystalline silicon structure. The surface of SiO_2 is hydrolyzed in contact with water containing solution and silanol groups then are formed. These groups might be positively charged, negatively charged, or appear at neutral state, dependent upon the pH of the solution. Varying surface charge at the interface of SiO_2 and the electrolyte affects the flat-band voltage of the electrolyte-oxide-semiconductor system thereby shifting the capacitance-voltage curves (37).

Other triglyceride detection methods involve successive enzymatic detection of glycerol produced by triglyceride hydrolysis using one or more reactions and are overlooked below.

Glycerol Detection Based on Consecutive Action of Several Enzymes

The group of the glycerol detection methods involves phosphorylation of glycerol with glycerol kinase (GlyK) in the presence of ATP to form glycerol-3-phosphate and ADP:

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GlyK, Mg}^{2+}} \text{Gglycerol-3-phosphate} + \text{ADP} \quad [2]$$

This reaction can be combined with several different subsequent reactions for glycerol-3-phosphate (Eq. (3)) or ADP detection (Eq. (5–7)). All further overlooked enzymatic glycerol determination methods are summarized in Table 2.

Glycerol-3-phosphate can be oxidized by oxygen (Eq. (3)), producing dihydroxyacetone phosphate and hydrogen peroxide, in the presence of glycerol phosphate oxidase GlyPO (5, 38, 39) or by glycerol-3-phosphate dehydrogenase (GlyPDH) to produce NADH (Eq. (4)):

$$\text{Glycerol-3-phosphate} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{GlyPO}} \text{Dihydroxyacetone-3-phosphate} + \text{H}_2\text{O}_2 \quad [3]$$

Subsequently, the concentration of H_2O_2 can be determined optically, using commercially available kits, or electrochemically. The oxygen consumption can be assayed also. Ferricianide was also used for the electrochemical determination of H_2O_2 produced in the enzymatic oxidation of glycerol (36). However, the ferri/ferrocenium redox couple is unstable and ferrocenium is easily re-oxidized by oxygen. In addition, these compounds can be easily diffused away from the electrode surface.

$$\text{Glycerol-3-phosphate} + \text{NAD}^+ \xrightarrow{\text{GlyPDH}} \text{Dihydroxyacetone-3-phosphate} + \text{NADH} \quad [4]$$

The conversion of NAD^+ to NADH can be detected spectrophotometrically at 340 nm or fluorometrically at 450 nm or amperometrically. The amount of NADH oxidized is stoichiometric to the amount of glycerol converted by glycerol kinase.

The method using a modified reagent kit for spectrophotometric hydrogen peroxide detection that contained higher amounts of enzymes GlyK, GlyPO and lipoprotein lipase for quantitative detection of triglycerides (TG) in serum lipoproteins and serum-free glycerol (FG) by high-performance liquid chromatography (HPLC) was reported (5). After separation of serum constituents using a new gel-permeation column, which eliminated non-specific adsorption of lipoproteins to TSK-gel materials, and a new eluent, TG and FG were spectrophotometrically detected at 550 nm by on-line reaction. HPLC patterns showed five peaks corresponding to chylomicrons, very-low-density (VLDL), low-density (LDL), high-density (HDL) lipoproteins and FG (5).

A flow injection chemiluminescent method for the determination of glycerol using a co-immobilized enzyme reactor containing glycerokinase and glycerol-3-phosphate oxidase is described (39). The hydrogen peroxide produced is monitored by using a luminol chemiluminescence reaction in the presence of catalyst such as Co(II). The method is applied for the determination of glycerol in blood serum produced off-line from triglycerides using lipase isolated from bovine pancreas (39).

A tri-enzyme system for glycerol determination can be prepared by using GlyK, pyruvate kinase (PK), and pyruvate oxidase (PO) (40) or lactate dehydrogenase (LDH) (8). Formed in PK catalyzed phospho-transfer reaction (Eq. (5)) pyruvate is oxidized with oxygen (Eq. (6)) by PO or by LDH (Eq. (7)):

$$\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow{\text{PK, Mg}^{2+}} \text{Pyruvate} + \text{ATP} \quad [5]$$

$$\text{Pyruvate} + \text{Phosphate} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{PO}} \text{Acetyl phosphate} + \text{HCO}_3^- + \text{H}_2\text{O}_2 \quad [6]$$

The consumption of oxygen through the PO catalyzed reaction can be monitored amperometrically.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+ \quad [7]$$

Evaluation of glycerol amount is based on the detection of NADH consumed from the decrease in absorbance at 340 nm.

An automated polyenzymatic procedure using enzymatic set of soluble GK, PK and LDH was evaluated for the measurement of micromolar levels of glycerol in human plasma (8). It was determined that high concentrations of bilirubin ($>85\ \mu$M) may interfere glycerol detection.

Nowadays, environment-friendly analytical procedures are very much appreciated, and a significant contribution to this aim is the generation of less environmentally unfriendly wastes. This requirement could be achieved by using immobilized enzymes. Compagnone et al. constructed biosensor for the measurement of glycerol in FIA using covalently immobilized GlyK and GlyPO enzymes (Eqs. (2–3)) in conjugation with a hydrogen peroxide sensitive electrode (38). Different immobilization strategies of the enzymes have been studied including random and asymmetric immobilization onto a polymeric support (nylon net, BSA-glutaraldehyde, cellulose acetate, polycarbonate membranes) and immobilization onto two different commercialized membranes (Immobilon and Biodyne). The most effective configuration was the GlyK immobilization on glass beds and packed to form a reactor while GlyPO was kept at the electrode surface immobilized onto preactivated Immobilon AV membrane. The working electrode of this analytical system was covered by enzyme enclosed within cellulose acetate and polycarbonate membranes and was allocated in a Universal Sensors flow-through electrochemical cell (38).

An amperometric tri-enzyme sensor for glycerol determination in wine was prepared by immobilizing GK, PK, and pyruvat oxidase (PO) on a polydimethylsiloxane coated Pt electrode (40). Tri-enzyme membrane was prepared from the mixture of

TABLE 2
Review of enzymatic detection methods for glycerol and triglyceride

Biomaterial	Detection method	Electrode material; electron acceptor; potential	Detection limits, S/N = 3; (selectivity)	Linear range	Stability	Reference
Enzymatic						
GalO	Amperometric	Oxygen electrode	(4 mV/mM)	2–200 mM	2 months	41
Lipase, GlyK, GlyPO	HPLC, with spectrophotometric detector ($\lambda = 550$ nm)	—	300 μM glycerol; 1.5 mM VLDL	0.2–2 mM glycerol and VLDL	—	5
GlyK, GlyPO	Amperometric	Pt(H_2O_2); +0.65 V vs Ag/AgCl	0.5 μM	0.002–2 mM	1 month	38
GlyK, PK, PO	Amperometric	Pt(H_2O_2) coated with PDMS; +0.4 V vs Ag/AgCl	5 μM	Up to 0.5 mM	10 days	40
GlyK, GlyPO	Chemiluminescence	—	7 μM	0.2–1 mM	—	39
GlyK, PK, LDH	Spectrophotometric ($\lambda = 340$ nm)	—	8 μM	Up to 250 μM	—	8
GlyDH/NAD, NADH oxidase	Chemiluminescence	—	0.07 μM	0.3–300 μM	—	42
Lipase, GlyDH/NAD	Spectrophotometric ($\lambda = 340$ nm)	—	—	50–250 mg/dl TG 0.005–10 mM glycerol	—	34
GlyDH/NAD	Spectrophotometric ($\lambda = 340$ nm)	—	22 μM	0.33–3.26 mM	2 month	45
GlyDH/NAD	Spectrophotometric ($\lambda = 340$ nm)	—	65 μM	22–109 mM	1 week	46
GlyDH/NAD	Spectrophotometric ($\lambda = 340$ nm)	—	87 μM	0.33–3.26 mM	2 weeks	10
Lipase, GlyDH/NAD, HRP	Amperometric	Oxygen electrode	50 μM in aqueous standards; 100 μM in serum samples; 300 μM TG	—	—	35
Lipase, GlyDH/NAD	Amperometric	Carbon electrode modified with adsorbed Meldola Blue, Nile Blue, Toluidine Blue O; 0 V vs Ag/AgCl	(2–9 nA/mM glycerol; 0.32 μA/mM trioleate)	Up to 1–10 mM glycerol; up to 0.75 mM trioleate	—	36

(Continued on next page)

TABLE 2
Review of enzymatic detection methods for glycerol and triglyceride *(Continued)*

Biomaterial	Detection method	Electrode material; electron acceptor; potential	Detection limits, S/N = 3; (selectivity)	Linear range	Stability	Reference
GlyDH/NAD	Amperometric	Carbon paste; NAD oxidation products; 0 V vs Ag/AgCl	0.43 μM (162 nA/mM)	1–100 μM	3 days	44
GlyDH/NAD	Amperometric	Graphite; +0.5 V vs Ag/AgCl	5 μM with K^+	0.02–0.2 mM with K^+; 0.04–0.4 mM without K^+	3 months	48
GlyDH-NAD	Amperometric	Polyaniline modified aluminum; hexacianoferrate; +0.4 V vs SCE	1 μM	0.005–2 mM	30 days	47
GlyDH-PQQ	Amperometric	Graphite modified with redox hydrogel modified with PVI complexed with osmium-DMBP +0.2 V vs Ag/AgCl	1 μM (32 mA/M $\times$ cm^2)	1–200 μM	20 hours	66
Bacterial						
Mutant bacteria from *Bacillus subtilis*	Potentiometric	Oxygen electrode	(103.2 mV/M)	0.11–1.09 M	18 days	16
Gluconobacter oxydans cells (GlyDH-PQQ)	Amperometric	Gold; osmium-PVI; +0.15 V vs Ag/AgCl	200 μM (0.065 μA/ mM $\times$ cm^2)	Up to 170 mM	68 hours (50%)	60
Lipase and *Gluconobacter oxydans* cells	Amperometric	Graphite; ferricyanide; +0.3 V vs SCE	20 μM glycerol (755 mA/mM glycerol; 58 mA/mM triolein)	Up to 2 mM glycerol; up to 12 mM triolein	7 and 3 days with and without stabiliser	33

GalO—galaktozoxidase; GlyK—glycerokinase; GlyPO—glycerolphosphate oxidase; PK—pyruvate kinase; PO—pyruvate oxidase; LDH—lactate dehydrogenase; HRP—horseradish peroxidase; GlyDH/NAD—NAD dependent glycerol dehydrogenase; GlyDH-PQQ—PQQ dependent glycerol dehydrogenase; TG—triglyceride; VLDL—very low density lipoproteins, PDMS—polydimethylsiloxane; PVI—polyvinylimidazole; DMBP—$(4,4'\text{-dimethylbipyridine})_2Cl$.

the photo-crosslinkable poly(vinyl alcohol) and enzyme solutions. The consumption of oxygen through the PO catalyzed reaction was monitored amperometrically at −0.4 V vs. Ag/AgCl. The permselectivity of the PDMS membrane made it possible to monitor the enzymatic oxygen consumption without interference by reducible species such as hydrogen peroxide, which is often used as a sterilizer of containers (bottles etc.) or produced during the oxidase-catalyzed reaction, and often persist in conserved food.

Glycerol detection can be also achieved by single enzyme catalyzed reaction.

Glycerol Detection Using Single Enzyme-Based Analytical Systems

Such detection principle of glycerol involves direct oxidation using glucose oxidase (GlyO), galactose dehydrogenase (GalO) NAD-dependent or PQQ-dependent glycerol dehydrogenases (GlyDH-NAD, GlyDH-PQQ) resulting in the production of dihydroxyacetone and H_2O_2 or reduced cofactors (NADH or $PQQH_2$).

Oxidases. Oxygen is a natural electron acceptor of oxidases, therefore glycerol oxidation using oxidases are dependent on oxygen concentration in analytical systems.

$$\text{Glycerol} + O_2 \xrightarrow{\text{GlyO}} \text{Dihydroxyacetone} + H_2O_2 \qquad [8]$$

As glycerol oxidase is not commercially available these methods are not widely used.

Vega et al. developed a biosensor combined with flow injection system for determination of galactoside conjugates and glycerol (41). The biosensor was based on the enzymatic reaction of galactose oxidase (GalO) using galactose, raffinose, lactose and glycerol as substrates. Galactose oxidase was adsorbed onto polymer matrix of epoxy-vinyl alcohol and disposed into Mobicol cartridge. GalO converts galactoside conjugates to galactohexodialdose conjugates and glycerol to glyceraldehyde with formation of hydrogen peroxide and consumption of oxygen:

$$\text{Glycerol} + O_2 \xrightarrow{\text{GalO}} \text{Glyceraldehyde} + H_2O_2 \qquad [9]$$

Sensitivity of the biosensor was higher for galactose followed by raffinose, lactose, and glycerol. The system needs to be regenerated with cupric ions to avoid enzyme inactivation due to loss of copper from the active site (41). Unfortunately, the potential of electrochemical oxidation of H_2O_2 is high, and many electroactive compounds can interfere with the amperometric determination of glycerol, so variation of dissolved oxygen in the carrier was estimated utilizing an amperometric oxygen probe. Additional disadvantage of oxidase based analytical systems is that oxygen usually competes with the artificial acceptor for binding sites of the enzyme and fluctuations of oxygen concentration often observed in biological liquids can distort results of analysis.

NAD-Dependent Dehydrogenases. Dehydrogenases are not sensitive to oxygen. NAD-dependent dehydrogenase based analytical systems are often used for analysis of glycerol (10, 34–36, 42–48). Although NAD-dependent dehydrogenase sensors are not dependent on oxygen the addition of the free-diffusing coenzyme NAD^+ is necessary.

Glycerol concentration in NAD-dependent glycerol dehydrogenase (Gly/NAD) catalyzed reaction is quantitatively related to NAD (NADH):

$$\text{Glycerol} + NAD^+ \xrightarrow{\text{GlyDH/NAD}} \text{Dihydroxyacetone} + \text{NADH} + H^+ \qquad [10]$$

Rate of conversion of NAD^+ to NADH monitored spectrophotometrically at 340 nm (10, 34 42, 46) or amperometrically (35, 36, 42, 47, 48).

Spectrophotometric NADH Detection. Masoom and Worsfold described automated flow injection manifold with stopped flow and merging zones for determination of triglycerides and glycerol (34). A single reagent cocktail is synchronously merged with sample and the rate of formation of NADH monitored spectrophotometrically. Flow analysis process is presented as profitable design allowing high data precision, high throughput and robustness. Addition advantages have been gathered by coupling a solid-phase reactor with immobilized enzyme (10, 42, 46). An aminopropyl derivatized controlled pore glass was used for enzyme immobilization very often (10, 42, 46). The development of automatic methods of analysis associated with enzyme immobilization could make the enzymatic assays cost effective. Flow injection analysis (FIA) method with parallel multi-site spectrophotometric detection for the sequential enzymatic determination of glycerol and ethanol in wines was reported (42). A 50-fold dilution of the samples before introduction into the system was necessary. Sequential injection analysis (SIA) is an attractive alternative to flow injection analysis because of its simplicity, flexibility and reduced reagent consumption. Despite of that this method is criticized for its low sampling frequency. Sequndo and Rangel presented the SIA system with increased throughput rate for spectrophotometric determination of glycerol and ethanol in wines (10). Since glycerol concentration in wines is higher than the response range for majority enzyme procedures based on spectrophotometric NADH detection, an off-line diluting step is required, thus causing an increase in the time consumed to carry out the analysis. An automatic flow procedure for spectrophotometric glycerol analysis in wine aimed at on-line sample dilution and less waste generation under the software control of the flow system network has been described (46).

NADH oxidase might be applied for oxidation of NADH:

$$\text{NADH} + H^+ + O_2 \xrightarrow{\text{NADH.oxidase}} NAD^+ + H_2O_2 \qquad [11]$$

The possibility to combine reactions presented in (Eqs. (10) and (11)) was shown and hydrogen peroxide formed was detected chemiluminometrically via a luminol-hexacyanoferrate (III) reaction in combination with flow injection analysis (42). Glycerol dehydrogenase and NADH oxidase were co-immobilized on poly(vinyl alcohol) beads and incorporated in a flow-injection system.

A simultaneous method for the determination of ethanol and glycerol in wines based on the coupling of pervaporation-chemical derivatisation-photometric detection for ethanol and biochemical derivatisation-fluorimetric detection for glycerol is proposed (43). The determination of glycerol is based on its oxidation by oxidized NAD^+ catalyzed by glycerol dehydrogenase immobilized on porous-glass, the reduced form of the coenzyme (NADH) being spectrofluorimetrically monitored ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 460$ nm) (43).

Amperometric NADH Detection. However the spectrophotometric detection is complicated if colored and non-transparent samples are investigated. To solve such problems electrochemical measurements might be applied. Moreover, it allows elimination some difficulties encountered in photometric detection, such as the overlap of absorbing species and turbidity.

The formal potential of the NAD/NADH couple is −540 mV vs. SCE. Theoretically, NADH should be easily oxidized at potentials near 0 V vs. SCE. Due to slow kinetics, however, direct oxidation of NADH at the electrode surface does not take place at significant rates below +400 mV. In addition, the direct oxidation of NADH at a solid electrode surface is irreversible and frequently results in electrode fouling. However, direct anodic oxidation of NADH has been reported. M. I. Promodis et al. constructed a flow injection analysis system incorporating amperometric detection and GlyDH/NAD reactor for estimating glycerol in alcoholic beverages such as wines, beers and champagne (48). NADH, the product of the enzymatic reaction, was detected with graphite working electrode at +0.5 V vs. Ag/AgCl. No fouling effect at electrode surface was observed. According to previous investigations the graphite electrodes proved to be very efficient with respect to stability, reproducibility and sensitivity for the determination of NADH (49). GlyDH/NAD was immobilized through chemical modification of several supporting materials such as aminopropyl and isothiocyanate controlled pore glass, aminopolystyrene resin and *m*-aminobenzyloxymethyl cellulose.

Due to high overpotential (0.4, 0.7 and 1V vs. SCE for carbon, Pt and Au electrodes, respectively) (47) and considerable dimerization, NAD/NADH will not be detected properly by direct electrochemical oxidation on a solid electrode surface. In this way, significant efforts have been given in the last few decades toward identification of materials, which can effectively overcome the kinetic barriers necessary for the electrochemical regeneration of NAD^+. A way to decrease high overpotential and minimize the side reactions is to use an appropriate catalyst for NADH electrooxidation (for example, ferricianide) that can improve electron-transfer kinetics:

$$\mathrm{NADH} + \mathrm{Fe(CN)_6^{3-}} \longrightarrow \mathrm{NAD^+} + \mathrm{Fe(CN)_6^{4-}} \qquad [12]$$

$$\mathrm{Fe(CN)_6^{3-}} \xrightarrow{+e^-(\text{electrode})} \mathrm{Fe(CN)_6^{4-}} \qquad [13]$$

Among the best-known structures, which can be active in the electrocatalytic oxidation of NADH *o*-quinones, *p*-quinones, and phenothiazine as well as phenoxazine derivatives are found. Nevertheless, most of the mediators are strongly pH dependent. The majority of commonly used mediators such as Meldola Blue, Nile Blue, *p*-benzoquinone, ferrocenes and Methylene Blue are inactive in alkaline media (>8). Ferricianide and dichlorophenolindophenol tested at pH 9.0 showed good electrochemical behaviour, but their analytical performance was limited in the absence of the enzyme diaphorase (48).

Another possibility to realize NADH amperometric detection can be executed by coupling with a second enzyme that regenerates the reduced NAD^+. Enzymatic regeneration of NAD^+ has been described using different schemes such as diaphorase, horseradish peroxidase (HRP), lipoamide dehydrogenase, or tyrosinase and salicylate hydroxylase. NADH indirectly monitored amperometrically using a membrane oxygen electrode according to the horseradish peroxidase (HRP) catalyzed reaction (35):

$$\mathrm{NADH} + \mathrm{H^+} + \frac{1}{2}\mathrm{O_2} \xrightarrow{\text{HRP, } Mn^{2+}} \mathrm{NAD^+} + \mathrm{H_2O} \qquad [14]$$

It was observed that the GlyDH reaction proceeded much more slowly than horseradish peroxidase 1, which adversely affected the analysis time and the sensitivity. This problem was determined by increasing the NAD concentration, maintaining the reaction at 33°C for 5 min. However, during incubation feedback inhibition by both NADH (competitively) and dihydroxyacetone (uncompetitively) took place, which resulted in a non-linear calibration graph (approximately a logarithmic response over wide concentration ranges) and reduced sensitivity compared with that of NADH-oxygen reaction (35).

Therefore, there is a great interest in the development of new materials or new immobilization strategies for already known electron-transfer mediators as shown by the number of publications on the subject. Thus, the most recent efforts to prepare modified electrodes that show electrocatalytic activity toward NADH oxidation have been focused on the use of electropolymerized films as new materials, either redox polymers (poly(*o*-aminophenol), poly(*o*-phenylenediamine) (42), polydimethylsiloxane(40)) or conducting polymers such as poly(aniline) (47, 50, 51). Potassium ferrocyanide and polyaniline modified Al electrode amperometric response to glycerol achieved at 0.4 V vs. SCE (47). Electrochemical oxidation of NADH using carbon electrodes modified with irreversibly adsorbed electron transfer mediators Toluidine Blue O, Nile Blue and Meldola Blue was realized at 0 V vs. saturated Ag/AgCl electrode (36, 52). GlyDH/NAD was adsorbed on the electrode entrapped in gelatin,

immobilized in polylysine gel, or trapped in two types of organic salts (36).

Alvarez-Gonzalez et al. detected that electrochemical oxidation of the adenine moiety in NAD^+ and other adenine nucleotides on carbon paste electrodes after anodic polarization at potentials above 1.2 V (vs. Ag/AgCl) in alkaline solutions gives rise to redox-active products, which are strongly adsorbed on the electrode surface. Carbon paste electrodes modified with the oxidation products of NAD^+show excellent electrocatalytic activity toward NADH oxidation, reducing its overpotential by about 400 mV (42). The catalytic oxidation current allows the amperometric detection of NADH at an applied potential of +50 mV (vs. Ag/AgCl). In such amperometric glycerol sensor NAD^+ acts not only as cofactor of the dehydrogenase enzyme but also as precursor of the NADH electrocatalytic system. The enzyme GlyDH and its cofactor NAD^+ were co-immobilized in a carbon paste electrode using an electropolymerized layer of nonconducting poly(*o*-phenylenediamine). Amperometric signals useful for glycerol detection were obtained at applied potentials as low as 0 V. Glycerol dehydrogenase suffers from the lack of selectivity. Moreover, other species that could be present in the sample may be oxidized at the working electrode potential. Citric, tartaric, and malic acids are generating amperometric signals lower than 5% of the corresponding one recorded for glycerol at the same concentration level. Significant interference was observed for 1,2-ethanediol and 1,2-propanediol (the signal was equal to the corresponding one registered for the same glycerol concentration) and ascorbic acid (twice higher as the signal for the same glycerol concentration) (42).

One important advantage of the enzymatic systems employing electrochemical detection schemes is that no interference from absorbent species and turbidity is usually observed.

PQQ-Dependent Dehydrogenases. The most promising tool for analysis is a relatively new class of enzymes pyrroloquinoline quinone (PQQ) dependent dehydrogenases. The main advantage of PQQ dependent enzymes as compared with pyridine nucleotide and flavin dependent are insensitivity to oxygen and redundant addition of the cofactor. Besides, some of them exhibit direct electron transfer between the active site and suitable electrode (15, 44, 53–56).

$$\text{Glycerol} + \text{M} \xrightarrow{\text{GlyDH-PQQ}} \text{Dihydroxyacetone} + \text{MH}_2 \quad [15]$$

There are only few publications on the properties of PQQ-dependent glycerol dehydrogenases. The first study on solubilization, purification and properties of membrane-bound PQQ dependent glycerol dehydrogenase from *Gluconobacter industrius* has been published in 1985 (57). The PQQ-glycerol dehydrogenase (GlyDH-PQQ) was defined as an enzyme that shows broad substrate specificity toward many kinds of polyhydroxyl alcohols including glycerol, D-mannitol, D-sorbotol, D-arabitol, adonitol, propylene glycol and *meso*-erythriol, but not ethanol, aliphatic aldehydes, D-glucose, D-fructose, D-gluconate or 2-keto-D-gluconate (57). Recent experimental studies of K. Matsushita and his group showed two different membrane-bound quinoproteins D-arabitol (ArDH) and D-sorbitol (SlDH) dehydrogenases purified from *Gluconobacter suboxydans* IFO 3257 and IFO 3255 respectively to be identical (58) and responsible for almost all sugar/alcohol oxidation in *Gluconobacter* species. All previously isolated quinoenzymes, such as glycerol dehydrogenase, polyol dehydrogenase, mannitol dehydrogenase, seem to be the same major polyol dehydrogenase as SlDH or ArDH. Both these enzymes including other so-called polyol dehydrogenases are able to recognize secondary alcohols with R-configuration in the sugar alcohols, in which D-gluconate is also included. ArDH is actually able to oxidize secondary alcohols such as 2-butanol, 3-pentanol, or 2,3-butanediol. The least unit that fulfils the essential polyol structure is glycerol in such sugar alcohols. Thus, the quinoprotein major polyol dehydrogenase, so far called as SlDH, ArDH, mannitol dehydrogenase, or polyol dehydrogenase, would be better called glycerol dehydrogenase (58).

The coexistence of many kinds of membrane-bound enzymes in the bacterial cytoplasmic membrane and separation of such co-solubilized enzymes, especially alcohol dehydrogenase, from glycerol dehydrogenase has been crucial for the investigation of glycerol dehydrogenase (57). Due to a low stability of the purified glycerol dehydrogenase, no analytical systems using this enzyme have been reported yet. Therefore, several groups of scientists suggested replacing enzymes with intact microorganisms.

Microbial Glycerol Determination Using Microbes Producing PQQ-Dependent Dehydrogenases. Microbial biosensors have several advantages over enzyme biosensors: the enzyme does not need to be isolated, enzymes are usually more stable in their natural environment in the cell, and coenzymes and activators are already present in the system. Especially favorable is the use of microbial biosensors for analysis of complex samples, containing many substrates, such as wastewater, starch hydrolysate and lignocellulose hydrolysate (35). A few reports dealing with bioconversion of glycerol to dihydroxyacetone by immobilized *Acetobacter pasteurianus* (59) and *Gluconobacter oxydans* (33, 60) cells containing membrane-bound PQQ-dependent glycerol dehydrogenase have been published.

Acetic acid bacteria are the predominant species that produce vinegar, and the activities of membrane-bound alcohol dehydrogenase and aldehyde dehydrogenase play a key role in efficient vinegar production by this means. Acetic acid bacteria have a variety of oxidoreductases, such as alcohol-, aldehyde, glucose-, and glycerol dehydrogenases, in the cytoplasmic membranes. In order to measure the substrate-oxidizing activity of intact cells of *Acetobacter pasteurianus* no. 2, a given amount of the bacterial cells was immobilized on a carbon-paste electrode and the current at the electrode was measured in a buffer solution. The electrode was prepared by packing the mixture of graphite powder and liquid paraffin into a glass tube and smoothing the surface on a weighing paper (61). When $Fe(CN)_6^{3-}$ was added

to the buffer solution, an anodic current was observed at +0.5 V vs. Ag/AgCl. When ethanol was added, the current started to increase to reach a steady-state condition within 3 min. The electrode was sensitive to acetaldehyde and lactic acid as well as to ethanol. Culture growing conditions affected the current response to various substances. The response of the electrode modified with the cells grown under static conditions was much higher than that of the electrode with the cells grown during shaking, and the electrode with ethanol-grown cells had a high response to ethanol and acetaldehyde compared with that of the electrode with glucose-grown cells (61). The present method can increase the number of viable cells within 1 h, including the time for immobilizing the bacterial cells on the electrode, which is much shorter than colony counting involving 3–7 days for colony formation on an agar plate. Carbon paste electrode modified with bacterial cells might be applied for the rapid and simple amperometric determination of cell activity, which is essential for the management of fermentation process.

Triglyceride assay based on triglyceride hydrolysis and glycerol detection using non-specific lipase isolated from *Candida rugosa* and intact *Gluconobacter oxydans* cells,' containing membrane-bound glycerol dehydrogenase, was developed. Here two approaches for the estimation of analyte concentration were applied: steady-state analysis of pre-hydrolyzed samples and a study of reaction kinetics (33).

Vostiar et al. described microbial biosensor sensitive to glucose, ethanol and glycerol. Biosensor was constructed for efficient electrical wiring of whole *Gluconobacter oxydans* cells using flexible polyvinylimidazole osmium functionalized polymer (60).

The main disadvantages of microbial biosensors: in many cases, they have low selectivity and rather long response time.

A bi-enzymatic biosensor for monitoring dihydroxyacetone production during oxidation of glycerol by bacterial cells of *Gluconobacter oxydans* is presented. Galactose oxidase oxidizes dihydroxyacetone efficiently producing hydrogen peroxide, which reacts with co-immobilized peroxidase and ferrocene pre-adsorbed on graphite electrode.

$$\text{Dihydroxy ace tone} + O_2 \xrightarrow{\text{GalO}} \begin{matrix} CH_2OH \\ | \\ CO \\ | \\ CHO \end{matrix} + H_2O_2 \qquad [16]$$

Dihydroxyacetone is considered to be a better substrate for galactose oxidase than galactose itself. GalO is an enzyme with the broad specificity. Thus, many compounds beside dihydroxyacetone can be oxidized including galactose and di- and oligosaccharides with D-galactose in the terminal position. Sensitivity of GalO to glycerol is only 0.2% of the sensitivity toward dihydroxyacetone (61).

The system for microcalorimetric determination of dihydroxyacetone using *Gluconobacter oxydans* cells immobilized in calcium pectate gel beads was developed by Navratil et al. Glycerol kinase and galactose oxidase were immobilized on the controlled pore glass and used for the determination of glycerol and dihydroxyacetone, respectively. This strongly aerobic microbial conversion is used for industrial production of dihydroxyacetone (62).

Towards Glycerol Biosensors Based on Purified and Semi-Purified PQQ-Dependent Dehydrogenases. Effective combination of electrochemistry and biochemistry is often applied in the design of electrochemical biosensors (63). Despite the fact that PQQ-dependent glycerol dehydrogenase (GlyDH-PQQ) has been described (57, 58), only few biosensors based on this enzyme have been previously designed (64–67). Biosensors have some advantages over other analytical techniques, e.g., low requirements for sample pre-treatment, easy operation, and low price. Membrane-bound GlyDH-PQQ was immobilized onto graphite electrode surface by cross-linking with glutaraldehyde (64–66) under specially elaborated soft conditions (68). As electron transfer mediator phenazine methosulphate was used. Response of biosensor was strongly interfered by glucose, sorbitol and mannitol and less interfered by fructose, methanol and dulcitol which can be neglected because the concentrations of those compounds are normally lower than those of glycerol in tested beverages (64).

The purification of GlyDH-PQQ was complicated and the yield and stability of the purified enzyme were low (65). Then alternative biocatalytic material—membranes of disrupted bacterial cells containing GlyDH-PQQ—were applied for the design of biosensors (69, 67). The application of bacterial membranes is more promising because the enzyme is present in the natural environment. However, the undesirable activity of intracellular and membrane enzymes will interfere with analytical signal and reduce selectivity of such analytical system.

The application of GlyDH-PQQ and other glycerol oxidizing enzymes might be very promising direction in glycerol detection. Here conducting polymers might be successfully applied as versatile immobilization matrix (70), which can be assembled over redox enzymes and form nanoparticles (71). To facilitate electron transfer polypyrrole can be functionalized by redox species like osmium-biphyridile complexes (67, 72), electrochemically polymerizeable ferocene derivatives (73) nafion (74) and soluble redox mediators (67, 75). Several approaches might be applied for immobilization of GlyDH-PQQ on the conducting polymer substrate, one based on the entrapment of GlyDH-PQQ within polymer film (46) or covalent attachment to the surface of conducting polymer (76). Different immobilization methods enable to form biosensors with different selectivity as it was previously show (64). This effect is mainly based on semi-permeability of this conducting polymer (77). Moreover, GlyDH-PQQ might be applied in the design of potentiometric sensors as it was shown for other PQQ-dependent enzymes (56) and other bioelectronic devices like biofuel cells (55). Electrogenerated layers of insoluble redox mediators (78)

are very promising for facilitation of electron transfer between GlyDH-PQQ and electrode.

CONCLUSIONS

Enhancing of already known enzymatic methods is based on continuous work in an automatic or semi-automatic manner with spectrophotometric or amperometric detection. These systems use the biocatalyst immobilized on various supports. Recent works have been mostly focused on new immobilization strategies such as dip-coated ion-exchanger polymers loaded with catalysts such as Toluidine blue O and Meldola blue or containing inorganic metal complexes, inclusion in self-assembled monolayers on platinum and gold electrodes, or inclusion in composite and sol-gel electrodes. Structural effect of the electrode on the electro catalysis has also been recognized, and a decrease in overpotential needed for NADH oxidation can be achieved by using single-crystal or thick-film gold electrodes or by electrochemical activation of carbon fiber electrode surfaces.

The application of PQQ-dependent dehydrogenases in glycerol detection is a new trend in this direction and seems to be very promising. However, low GlyDH-PQQ stability problem still exists. One of the ways to solve this problem is application of GlyDH-PQQ containing cells, cell membranes or low purified enzyme, but this way significantly reduces the selectivity of analytic system. The next way is to find more convenient immobilization methods for GlyDH-PQQ, here the solution might be found by application of more protein friendly environments such as sol gels or conducting polymers.

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