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A dual fluorophore system for simultaneous bioassays

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Abstract

A detection scheme for the simultaneous evaluation of two bioassays based on fluorescence spectroscopy is presented. For the determination of hydrogen peroxide-generating enzymes or peroxidases, the non-fluorescent 4-(*N*-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH) is converted to the strongly fluorescent 4-(*N*-methylamino)-7-nitro-2,1,3-benzooxadiazole (MNBDA). Phosphatases are detected based on the cleavage of the non-fluorescent 5-fluorosalicyl phosphate (5-FSAP) under formation of the fluorescent 5-fluorosalicylic acid (5-FSA). While excitation of the fluorophores may be carried out at the same wavelength, their emission spectra differ significantly. This allows the read-out of both assays using commercially available microplate readers without additional chemometric tools. Compared with individual assays, limits of detection are similar, and linearity of the calibration functions for both enzymes is observed over 2–3 concentration decades starting at the limit of quantification. The simultaneous determination of glucose oxidase and acid phosphatase in honey is presented as example for the application of the detection scheme.

Keywords: Bioassays; Fluorescence; Simultaneous determinations; Glucose oxidase; Peroxidase; Phosphatase

1. Introduction

In bioanalysis, the determination of various enzymatic activities is one of the most important tasks [1]. Natural products are screened with respect to the presence of biocatalysts [2] and enzymes are also used as markers for signal amplification in several types of bioassays, including enzyme-linked immunosorbent assays (ELISA) [3]. In this field, they have partly replaced other sensitive detection schemes, for example radioimmunoassays [4]. Large series of synthetic substrates have been introduced for the most important enzymes, and in most cases, they are chromogenic or fluorogenic [5–11]. While chromogenic substrates are popular because only readily available instrumentation is required, fluorogenic substrates typically allow obtaining lower limits of detection [9]. To achieve higher sample throughput, it is desirable to miniaturize assay formats, to increase the speed of analysis and to obtain more information within a single assay.

Simultaneous bioassays are a possibility to increase the degree of analytical information that can be gathered from a sample within a short time. Important aspects are low limits of detection, easy handling and readily available instrumentation. Currently, several different approaches to achieve this goal are described or are under development. Chelates of europium(III), terbium(III), dysprosium(III) and samarium(III), all of which are characterized by intense long-lived fluorescence, have been proposed as markers for immunoassays, in which up to four analytes can be determined simultaneously [12]. However, the quantum yield varies strongly between these complexes, time-resolved fluorescence should be used to fully exploit the potential of this method and the determination of enzymatic activity is difficult. Although the enzyme-amplified lanthanide luminescence (EALL) [13], a detection scheme in which a substrate is converted to a product, which can transfer excitation energy to the lanthanide cations, has been described in recent years, a simultaneous enzymatic activity determination based on this approach has not been introduced yet and will be difficult to achieve due to the special requirements of these methods. Luminescent nanoparticles based on semiconductor materials ("quantum dots") [14–17] or on dyes entrapped in polymer materials [18,19] have also been proposed as biolabels with multiple colours for affinity assays [17,20], thus resulting in the possibility of multianalyte measurements. However, there is

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currently no means to change their fluorescent properties by an enzymatic conversion, so that they cannot be used in enzyme assays.

Therefore, we have set up a simple fluorescence-based detection scheme for two-analyte enzyme assays, which should be carried out with readily available instrumentation and without the use of chemometric tools.

2. Experimental

2.1. Reagents

All chemicals were purchased from Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany) and Fluka (Neu-Ulm, Germany). The enzymes glucose oxidase GOD (E.C. 1.1.3.4), alkaline phosphatase aP (E.C. 3.1.3.1), acid phosphatase acP (E.C. 3.1.3.2) and horseradish peroxidase POD (E.C. 1.11.1.7) as well as microperoxidase MP-11 (from equine heart cytochrome *c*) were purchased from Sigma (Deisenhofen, Germany).

2.2. Apparatus

All quantitative fluorescence determinations were performed with a FLUOstar microplate reader from BMG LabTechnologies (Offenburg, Germany) with FLUOstar software version 2.10-0. For the measurements, four different filters were used: 320 nm (bandwidth ±20 nm) and 470 nm (bandwidth ±15 nm) filters for excitation and 405 nm (bandwidth ±25 nm) and 545 nm (bandwidth ±10 nm) filters for emission. The measurements were performed on Corning (Costar No. 3915, black) 96-wells micro-titration plates, which were purchased from Diagonal (Münster, Germany). For photometric measurements on microplates, a Spectra Max 250 Reader from Molecular Devices (Sunnyvale, CA) with SOFTmax PRO software version 1.2.0 was used. The transparent flat form 96-wells micromethod plates were purchased from Emergo BV (Landsmeer, The Netherlands).

Fluorescence spectra were recorded using an Aminco Bowman AB2 luminescence spectrometer from Polytec (Waldbronn, Germany) with software version 5.00. The excitation and emission spectra of all fluorophores were recorded for 10^{-4} M solutions in acetonitrile.

2.3. Synthesis

The synthesis of 4-(*N*-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH) was performed as described in literature [21]. MNBDH has recently become commercially available by Molecular Probes (Eugene, OR).

5-Fluorosalicyl phosphate (5-FSAP) was synthesized as described by Evangelista et al. [22].

2.4. Enzymatic assays

2.4.1. Glucose oxidase (GOD) assay [23]

Fifty microliters of a solution of GOD (10^{-4} to 0.1 u mL^{-1}) in acetate buffer (pH 5.5; 0.01 M) were pipetted to 100 μL of a glucose solution (2×10^{-3} mol L^{-1}). 1.08 mg MNBDH were dissolved in 10 mL acetonitrile, and 1.4 mL of this solution were added to a solution of 2.64 mg POD (40 000 u L^{-1}) in 10 mL phosphate buffer (pH 5.8; 0.01 M). Forty microliters of this mixture were pipetted after 15 min to the GOD solution. After incubation at room temperature (15 min), the fluorescence was measured at excitation and emission wavelengths of 470 and 545 nm, respectively.

2.4.2. Alkaline phosphatase (aP) assay [13]

Fifty microliters of a solution of 2.36 mg 5-FSAP dissolved in 10 mL acetonitrile were pipetted to 50 μL of an aP solution (1×10^{-4} to 0.1 u mL^{-1}; Tris buffer; pH 8.5; 0.1 M). One hundred and fifty microliters water (150 μL) was added and after 15 min the fluorescence was measured at excitation and emission wavelengths of 320 and 405 nm, respectively.

2.4.3. Acid phosphatase (acP) assay [24]

Fifty microliters of a solution of 2.36 mg 5-FSAP dissolved in 10 mL acetonitrile were pipetted to 50 μL of an acP solution (0.02–1 u mL^{-1}; citrate buffer; pH 5; 0.02 M). Fifty microliters of water were added and after 15 min the fluorescence was measured at excitation and emission wavelengths of 5-FSA.

2.4.4. Peroxidase (POD) assay [25]

One milliliter of a solution of 1.08 mg MNBDH dissolved in 10 mL acetonitrile was added to 25 mL of a 10 mM solution of H_2O_2. Two hundred μL of this mixture were pipetted to 50.7 μL of a solution of POD (0.02 to 10 u mL^{-1}) in Tris buffer (pH 7.5; 0.1 M). After the sample had been incubated for 15 min at room temperature, the fluorescence was measured at excitation and emission wavelengths of MNBDA.

2.4.5. Microperoxidase (MP-11) assay

1.08 mg MNBDH was dissolved in 10 mL acetonitrile and 1.2 mL of this solution were added to 10 mL of a 30 mM solution of H_2O_2. Sixty-five microliters of this mixture were pipetted to 100 μL of a solution of MP-11 (1×10^{-10} to 1×10^{-7} mol L^{-1}) in Tris buffer (pH 7.5; 0.1 M). After the sample was incubated for 15 min at room temperature, the fluorescence was measured at the wavelengths of MNBDA.

2.4.6. Simultaneous enzymatic assays

The assays of the enzymes GOD and aP, GOD and acP as well as aP and MP-11 were performed simultaneously. In all cases, the respective reactions were executed on one microplate by varying the concentrations of the two analytes in different directions. For each concentration of the analytes,

multiple determinations were made, in each well of the microtiterplate with a different concentration of the second analyte. The fluorescence of the respective products was read out subsequently. In the case of the parallel determination of GOD and aP and of GOD and acP, it was even possible to excite the fluorescent products with one single wavelength.

2.5. GOD and acP determination in honey

For the individual determination of acP and GOD and the simultaneous determination of the two enzymes in a honey sample, 2 g honey was dissolved in 5 mL citrate buffer (pH 4.8; 0.02 M). All enzyme measurements were dissolved by means of standard addition for calibration. Therefore, 35 μL honey solution were added to the three reaction solutions.

In order to verify the results, two reference reactions were performed. Determination of GOD with 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) [23]: 35 μL of the honey solution were pipetted to a solution consisting of 50 μL glucose (2×10^{-3} mol L^{-1}) and 25 μL GOD (0 u mL^{-1} and 0.001 to 0.1 u mL^{-1}; citrate buffer). After 15 min, 40 μL of acP (0 u mL^{-1} and 0.05 to 1 u mL^{-1}; citrate buffer), 30 μL POD/MNBDH solution (see above) and 100 μL citrate buffer were added. After a reaction time of 1 h, the reaction product was photometrically determined at a wavelength of 405 nm.

Determination of acP with *p*-nitrophenyl phosphate (NPP) [24]: 35 μL of the honey solution were pipetted to a solution consisting of 50 μL H_2O and 25 μL GOD (0 u mL^{-1} and 0.001 to 0.1 u mL^{-1}; citrate buffer). Afterwards, 40 μL of acP (0 u mL^{-1} and 0.05 to 1 u mL^{-1}; citrate buffer), 30 μL citrate buffer and 100 μL substrate solution (0.204 g NPP dissolved in 100 mL buffer) were added. After a reaction time of 1 h, the reaction was stopped by adding 40 μL NaOH (0.5 M). The product was photometrically determined at a wavelength of 410 nm.

3. Results and discussion

Goal of this work was the development of a detection scheme for simultaneous bioassays based on fluorescence spectroscopy. This should meet the following criteria:

- The detection of at least two different (groups of) enzymes (e.g., an oxidoreductase as peroxidase and a hydrolase as alkaline phosphatase) should be possible from a single reaction solution without interferences between the two assays.
- The analytical figures of merit (limit of detection, limit of quantification, linear range for calibration) should be similar to those of the individual assays.
- To allow the immediate application in routine laboratories, commercially available instrumentation (microplate fluorescence spectrometers, standard filters) should be used and no chemometric tools should be required.

Table 1
Fluorescence excitation and emission maxima for selected fluorophores

	Excitation maximum (nm)	Emission maximum (nm)
7-Amino-4-(trifluoromethyl)coumarin	361	492
5-FSA	313	418
7-Hydroxycoumarin	345	453
6-Nitro-3,4-benzocoumarin	395	479
7-Methoxycoumarin	346	391
Fluorescein	468	518
Diflunisal	313	420
MNBDA	468/335	522

3.1. Selection of the fluorophores

To obtain assay conditions, which allow low limits of detection and a linear range of the assay over at least two decades of concentration, the selection of the fluorophores was based on some additional criteria:

- Upon enzymatic conversion, non-fluorescent substrates should be converted to strongly fluorescent products.
- To be able to detect two or more fluorophores simultaneously, without using mathematical tools like chemometrics, the emission bands should not overlap significantly.
- The substrates should be stable during storage over a reasonable period.
- The enzymatic conversions and, if possible, the detection should be carried out at or close to physiological pH.

A series of fluorophores consisting of coumarin, fluorescein, salicylate and 4-nitrobenzooxadiazole (NBD) backbones was investigated with respect to their excitation and emission maxima. These data are provided in Table 1. A detailed comparison of all excitation and emission spectra led to the conclusion that only few combinations could meet all criteria mentioned above. Only a combination of 5-FSA or diflunisal with fluorescein or MNBDA would in principle be possible. Finally, 5-FSA and MNBDA were selected for the following reasons: under the conditions investigated 5-FSA is a three-fold stronger fluorophore than diflunisal. MNBDA is characterized by a second excitation maximum, which shows some overlap with the excitation maximum of diflunisal or 5-FSA. This is presented in Fig. 1 and could enable the use of a common excitation wavelength for both compounds. The emission spectra of 5-FSA and MNBDA overlap only slightly, and with the use of appropriate emission filters, chances to obtain no cross-selectivity into both directions appeared to be good for this pair of compounds.

3.2. Simultaneous detection of 5-FSA and MNBDA

The conditions for the simultaneous detection of the two fluorophores 5-FSA and MNBDA were investigated in the following. Both fluorophores were determined simultaneously, and concentrations were varied. In Table 2, the

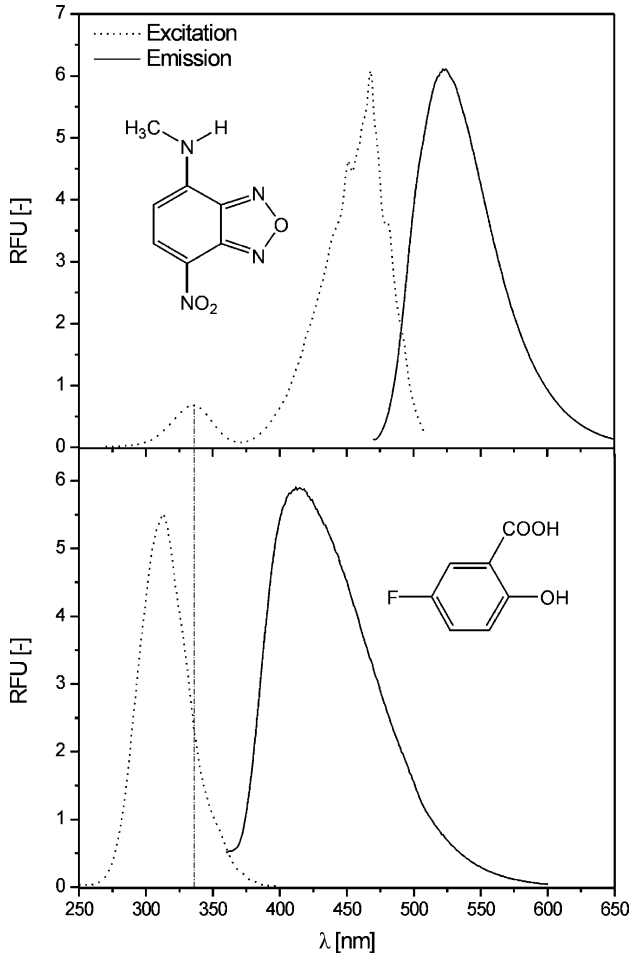

Fig. 1. Fluorescence excitation and emission spectra of MNBDA (above) and 5-FSA (below).

detection wavelengths for the filter-based microplate spectrofluorimeter and the limits of detection for both substances obtained in the individual measurement (both compounds in different wells of the microplate) and in the simultaneous determination (both compounds in one common well of the microplate) are provided. For both approaches and both compounds, the limits of detection are identical with 10 nmol L^{-1}, thus proving that there are no interferences of one fluorophore with respect to the other from the detection point of view.

3.3. Enzyme detection schemes

The enzymatic reactions, which are carried out with MNBDH and 5-FSAP are presented in Fig. 2. Glucose oxidase catalyzes the oxidation of glucose by oxygen under formation of gluconolactone and hydrogen peroxide. The latter converts, in the presence of peroxidase as catalyst, the non-fluorescent MNBDH to the fluorescent MNBDA. The non-fluorescent 5-FSAP is hydrolyzed under catalysis of phosphatase to the fluorescent 5-FSA.

Despite the excellent fluorescent properties of MNBDA, its peroxidase-catalyzed formation is comparably slow. For this reason, the substrate is only applicable for those cases, where POD can be used in higher concentrations, e.g., to detect hydrogen peroxide formed in a previous reaction step. Preliminary investigations, however, showed that the microperoxidase MP-11 rapidly catalyzes the conversion of MNBDH to MNBDA by hydrogen peroxide and that MNBDH can therefore be considered to be an excellent new substrate for this microperoxidase.

The limits of detection were optimized for POD and MP-11. As is obvious from Table 3, the limits of detection of MP-11 are improved by more than a factor of 100 compared with POD. While only 17 nmol L^{-1} are achieved as LOD for POD, 100 pmol L^{-1} are observed for MP-11. Linear ranges of the calibration functions are observed from 5 × 10^{-8} to 2 × 10^{-6} mol L^{-1} for POD and from 3 × 10^{-10} to 1 × 10^{-7} mol L^{-1} for MP-11. The limits of quantification are 5 × 10^{-8} and 3 × 10^{-10} mol L^{-1}. The relative standard deviations ($n = 8$) were determined for two different concentrations of POD and MP-11 each. As expected, the RSDs for POD and MP-11 are much lower for the higher

Table 2
Limits of detection for the individual and the simultaneous determination of MNBDA and 5-FSA

	λ_{ex} (nm)	λ_{em} (nm)	Individual determination (Ind) (×10^{-8} mol L^{-1})		Simultaneous determination (Sim) (×10^{-8} mol L^{-1})		Linear range (mol L^{-1})	RSD (%) (1 × 10^{-7} mol L^{-1})		RSD (%) (1 × 10^{-5} mol L^{-1})	
			LOD	LOQ	LOD	LOQ		Ind	Sim	Ind	Sim
MNBDA	468	524	1	3	1	3	3 × 10^{-8} to 1 × 10^{-5}	3.0	2.4	1.0	1.3
5-FSA	313	415	1	3	1	3	3 × 10^{-8} to 1 × 10^{-5}	5.0	8.7	1.0	2.0

Table 3
Limits of detection and relative standard deviations ($n = 8$) for multiple analysis of POD and MP-11 solutions at selected concentrations

Method	LOD (mol L^{-1})	LOQ (mol L^{-1})	Linear range (mol L^{-1})	RSD (%) (2 × 10^{-7} mol L^{-1})	RSD (%) (2 × 10^{-6} mol L^{-1})	RSD (%) (2 × 10^{-10} mol L^{-1})	RSD (%) (5 × 10^{-8} mol L^{-1})
POD	2 × 10^{-8}	5 × 10^{-8}	5 × 10^{-8} to 2 × 10^{-6}	15.8	5.3		
MP-11	1 × 10^{-10}	3 × 10^{-10}	3 × 10^{-10} to 1 × 10^{-7}			11.3	2.2

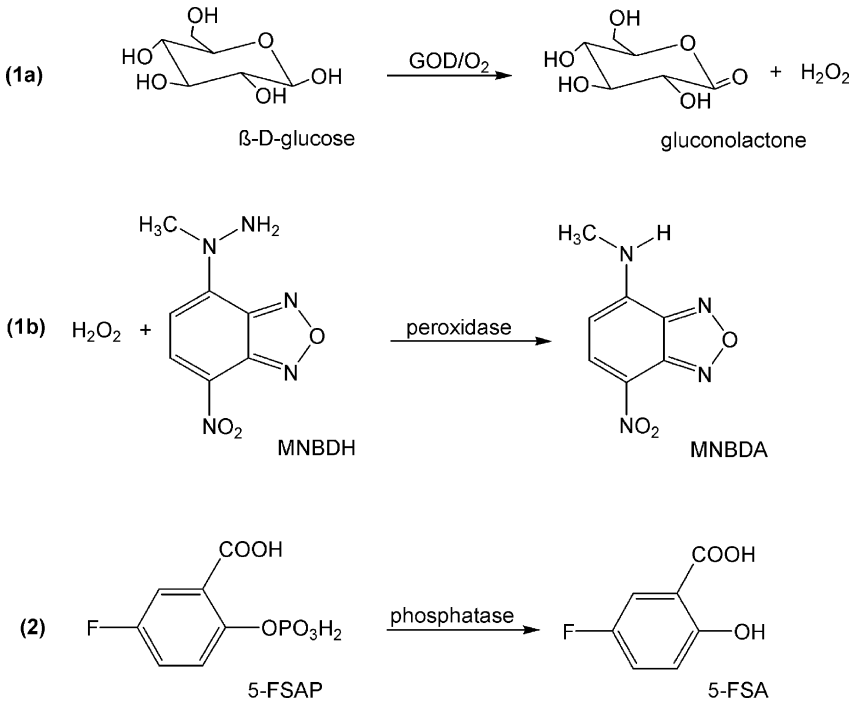

Fig. 2. Enzymatic reactions for the determination of GOD, POD and MP-11 using MNBDH and for the determination of phosphatases using 5-FSAP.

Table 4
Limits of detection for the individual and for the simultaneous determination of selected enzymes based on their reactions with MNBDH and 5-FSAP

Method	Individual determination LOD	Individual determination LOQ	Simultaneous determination with	LOD	LOQ
GOD	5×10^{-4} u mL^{-1}	2×10^{-3} u mL^{-1}	aP ($\lambda_{ex} = 320$ nm)	1×10^{-3} u mL^{-1}	3×10^{-3} u mL^{-1}
			aP	5×10^{-4} u mL^{-1}	3×10^{-3} u mL^{-1}
			acP	5×10^{-4} u mL^{-1}	3×10^{-3} u mL^{-1}
aP	5×10^{-4} u mL^{-1}	2×10^{-3} u mL^{-1}	GOD	5×10^{-4} u mL^{-1}	3×10^{-3} u mL^{-1}
			MP-11	5×10^{-4} u mL^{-1}	3×10^{-3} u mL^{-1}
acP	5×10^{-2} u mL^{-1}	2×10^{-1} u mL^{-1}	GOD	5×10^{-2} u mL^{-1}	2×10^{-1} u mL^{-1}
MP-11	1×10^{-10} mol L^{-1}	3×10^{-10} mol L^{-1}	aP	2×10^{-10} mol L^{-1}	6×10^{-10} mol L^{-1}

concentrations. In general, the RSDs for MP-11 are slightly lower compared with those for POD.

3.4. pH-optimization for the simultaneous determinations

Different enzymatic reactions may demand different pHs for the optimum performance. Therefore, assay conditions had to be optimized for the simultaneous enzyme–substrate reactions. The GOD assay is usually performed at pH 5.5–6, whereas the aP-catalyzed reaction should be carried out in the alkaline pH range from 9 to 10. In order to find a pH which is acceptable for both reactions, the individual assays were performed with a pH variation in 0.5 steps between pH 5.5 and 9.5, resulting in pH 7.5 as a compromise for both reactions. The parallel determination of aP and MP-11 was tested at pH 8.5 in comparison to pH 7.5, with better results being obtained in the latter case. The simultaneous measurements of acP and GOD were carried out at pH 4.8, the optimum pH value for acP, which was also acceptable for the GOD-catalyzed reaction.

Table 5
Concentrations of GOD and acP determined in a honey sample with the newly developed method and with reference methods

Method	Individual determination (u mL^{-1})	RSD (%) ($n = 5$)	Simultaneous determination (u mL^{-1})	RSD (%) ($n = 5$)
GOD ($\lambda_{ex} = 470$ nm)	28.6	6.0	27.1	12.9
GOD ($\lambda_{ex} = 320$ nm)	29.3	11.5	29.2	14.7
GOD Ref$_{ABTS}$	27.6	5.0		
acP	382.5	10.0	411.0	13.3
acP Ref$_{NPP}$	378.9	8.8		

3.5. Simultaneous enzyme determination in solution

The simultaneous two-enzyme determination was investigated for different couples of enzymes, as summarized in Table 4. In most cases, the limit of detection is identical for the individual and the parallel determination. There is no interference of one enzymatic reaction with respect to the other. Only the limit of detection of MP-11 in the simultaneous measurement with aP increases by a factor of 2 in comparison to the single determination. Expectedly, the limit of detection of GOD in the simultaneous determination with aP by exciting the fluorescent products of both assays with one single excitation wavelength increases also by a factor of 2. MNBDA was in this case excited at its lower excitation maximum (compare with Fig. 1).

3.6. Simultaneous enzyme detection in honey

Honey is known to contain different enzymes in significant concentrations, including acid phosphatase and glucose oxidase. Therefore, honey is well-suited to investigate the applicability of the simultaneous detection scheme for the analysis of real samples. To validate the results, enzyme detection systems based on established chromophores were used in parallel with the individual and the simultaneous determination based on the new method described in this paper. Calibration was performed based on standard addition for all newly developed and reference methods, as external calibration could not be performed for any of the methods due to matrix interferences. The results plus the respective RSDs ($n = 5$) for a honey sample are presented in Table 5. For GOD as well as for acP determination, all data obtained correlate well under consideration of the RSDs.

4. Conclusions

The simultaneous dual-enzyme determination of GOD or MP-11 and aP or acP has been demonstrated. In case of aP and GOD it has also been shown that it is possible to excite MNBDA and 5-FSA with one single excitation wavelength by yielding only a slightly higher LOD for GOD in comparison to two individual optimum excitation wavelengths. MNBDH has been introduced as an excellent new substrate for MP-11, and its combination with 5-FSAP as phosphatase substrate allows the simultaneous determination with only slightly reduced performance data compared with the individual determination. The applicability of the method was demonstrated for a honey sample containing GOD and acP. The possibility for expansion of this detection scheme to other enzymes, e.g., other oxidases as xanthine oxidase, is indicated by these results. However, similar reaction products have to be generated in these cases. Future work shall be directed towards the development of a two-analyte immunoassay and to a simultaneous two-enzyme post-column detection system for liquid chromatographic separations.

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