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An analysis of avidin, biotin and their interaction at attomole levels by voltammetric and chromatographic techniques

Received: 15 October 2004 / Revised: 7 December 2004 / Accepted: 10 December 2004 / Published online: 4 March 2005
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Abstract The electroanalytical determination of avidin in solution, in a carbon paste, and in a transgenic maize extract was performed in acidic medium at a carbon paste electrode (CPE). The oxidative voltammetric signal resulting from the presence of tyrosine and tryptophan in avidin was observed using square-wave voltammetry. The process could be used to determine avidin concentrations up to 3 fM (100 amol in 3 μ l drop) in solution, 700 fM (174 fmol in 250 μ l solution) in an avidin-modified electrode, and 174 nM in a maize seed extract. In the case of the avidin-modified CPE, several parameters were studied in order to optimize the measurements, such as electrode accumulation time, composition of the avidin-modified CPE, and the elution time of avidin. In addition, the

avidin-modified electrode was used to detect biotin in solution (the detection limit was 7.6 pmol in a 6 μ l drop) and to detect biotin in a pharmaceutical drug after various solvent extraction procedures. Comparable studies for the detection of biotin were developed using HPLC with diode array detection (HPLC-DAD) and flow injection analysis with electrochemical detection, which allowed biotin to be detected at levels as low as 614 pM and 6.6 nM, respectively. The effects of applied potential, acetonitrile content, and flow rate of the mobile phase on the FIA-ED signal were also studied.

Keywords Avidin · Biotin · Avidin–biotin technology · Square-wave voltammetry · Modified electrode · High-performance liquid chromatography · Diode array detection · Electrochemical transfer technique · Carbon paste electrode · Drug analysis

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Introduction

Biotin (*cis*-hexahydro-2-oxo-1-H-thieno-[3,4]-imidazoline-4-valeric acid), commonly known as vitamin H, is involved in the metabolism of amino acids and carbohydrates in organisms [1]. A low biotin intake has been reported to result in serious biochemical disorders in animal organisms, such as a reduced carboxylase activity, inhibition of protein and RNA syntheses, reduced antibody production, and other metabolic abnormalities [1–3]. Therefore, it is very important to monitor biotin levels in biological fluids as well as in food and food supplemental products, which constitute the main source of biotin for humans. A few analytical methods have been developed for the direct detection of biotin in pharmaceutical preparations, including high performance liquid chromatographic procedures coupled with different types of detectors (diode array, mass, electrochemical, and others) [2, 4–7].

Avidin, a minor constituent of egg white from reptiles, amphibians and birds, is a glycosylated and positively charged protein (at neutral pH) which usually forms tetramers [8, 9]. There are only a few papers that describe the direct detection of avidin, and those deal primarily with the levels in eggs. On the other hand, avidin combines selectively with biotin. The vitamin has a very high affinity to avidin (dissociation constant of 10^{-15} M), and this interaction has been utilized in many types of avidin-biotin technologies, such as immunohistochemistry, electron microscopy, enzyme-linked immunoassay, DNA hybridization, and construction of biosensors [10–18]. In an application that makes use of avidin-biotin binding, it is essential to establish methods for detecting and evaluating the interaction. The most commonly-used methods for detecting and evaluating avidin-biotin interactions are ELISA, fluorimetry, and some electrochemical methods [19–27].

To ensure that sufficient food is available for an expanding human population, it is necessary to exploit a number of new technologies that may help to increase production of agricultural foodstuffs. This production is heavily suppressed by bacterial or viral diseases, and also damage caused by insect pests. These undesirable processes can be prevented by using chemical compounds such as pesticides [28]. Use of these compounds, however, can result in environmental contamination, and they can also enter into the food chain. New molecular biology techniques are helping researchers to utilize natural bio-cidal compounds produced by plants that can protect them against pests [29, 30]. Recently, it was reported that avidin is toxic to a broad spectrum of lepidopteran, coleopteran, and dipteran pests of grains, fruits, and vegetables [31–34]. For the purposes of recombinant avidin production and biological protection of grain, a transgenic avidin corn plant was therefore developed.

There is much evidence demonstrating that a carbon paste electrode (CPE) is a suitable tool for use in biosensors [16, 35–47]. Moreover, carbon paste electrodes can be easily modified by adding different substances in order to increase sensitivity, selectivity and rapidity of determination [14, 40, 48, 49]. We primarily focused on utilization of these electrodes (CPE and modified CPE) in combination with square-wave voltammetry and adsorptive transfer stripping techniques for measuring nanogram quantities of avidin.

In this paper we describe the preparation of avidin- and avidin extract-modified CPEs for sensitive avidin detection in solution and in transgenic maize plants. We also describe the detection of biotin by an electrochemical method that utilizes the strong avidin-biotin interaction. In addition, we optimized and applied hyphenated analytical techniques for the detection of avidin, biotin and avidin-biotin interactions. We used high performance liquid chromatography coupled with a diode array detector (HPLC-DAD) to detect biotin, and flow injection analysis (FIA) with electrochemical detection (ED) to detect avidin and/or avidin-biotin interactions.

Experimental

Chemicals

Avidin, biotin, trifluoroacetic acid, carbon powder, sodium chloride, sodium citrate, hydrochloric acid, sodium carbonate, sodium acetate, acetic acid, and mineral oil were purchased from Sigma Aldrich Chemical Corp. (St. Louis, USA). HPLC-grade acetonitrile and methanol (>99.9%; v/v) were from Merck (Darmstadt, Germany). Flavone and all other reagents of ACS purity were purchased from Sigma Aldrich. Solutions were prepared using ACS water from Sigma Aldrich. The stock standard solutions of avidin and biotin at $1 \mu\text{g ml}^{-1}$ were prepared and stored in the dark at 4°C . All solutions were filtered through a $0.45 \mu\text{m}$ Teflon membrane filters (MetaChem, Torrance, CA, USA) prior to HPLC separations.

Measuring pH and absorption spectra

The pH was measured using a WTW inoLab Level 3 instrument (Weilheim, Germany), controlled by a personal computer program (MultiLab Pilot; Weilheim, Germany). The pH electrode (SenTix H, pH 0–14/0–100 $^\circ\text{C}/3 \text{ mol l}^{-1} \text{ KCl}$) was regularly calibrated using a set of WTW buffers (Weilheim, Germany). UV absorption spectra of avidin were determined using a diode array spectrophotometer (Hewlett-Packard model 8452A).

Electrochemical measurements

Electrochemical measurements were performed using an Autolab analyzer (EcoChemie, The Netherlands) in connection with a VA-Stand 663 (Metrohm, Zurich, Switzerland). The electrode system consisted of a carbon paste working electrode, an Ag/AgCl/3 M KCl reference electrode, and a platinum wire counter electrode. Acetate buffer (0.1 M CH_3COOH + 0.1 M CH_3COONa , pH 4.0) was used as the supporting electrolyte. Adsorptive transfer stripping square wave voltammetry (AdTS SWV) was performed using the following parameters: initial potential = 0.1 V, end potential = 1.3 V, amplitude = 25 mV, step potential = 5 mV, and frequency = 200 Hz. All experiments were carried out at 25°C . The raw data were treated using the Savitzky and Golay filter (level 2) and a moving average baseline correction (peak width = 0.05 mV) of the GPES software.

Preparation of CPE and avidin-modified CPE

The carbon paste (about 0.5 g) was made of graphite powder (Aldrich) and mineral oil (Sigma; free of DNase,

RNase, and protease). The ratio of the graphite powder and mineral oil was tested (see “Results” and “Discussion” sections). This paste was housed in a Teflon body with a 2.5-mm-diameter disk surface. Prior to measurements, the electrode surface was renewed by polishing with a soft filter paper. The surface was then ready to take measurements in a sample volume of 3–6 μl . The avidin-modified CPE was prepared in the same way as described above, with the addition of avidin to the CPE.

Flow injection analysis with CoulArray electrochemical detector

The flow injection analysis with electrochemical detection (FIA-ED) system consisted of two solvent delivery pumps operating over a range of 0.001–9.999 ml min^{-1} (Model 582 ESA Inc., Chelmsford, MA, USA), a reaction loop of 1 m length, and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector included two low volume flow-through analytical cells (Model 6210, ESA, USA). Each analytical cell consisted of four carbon porous working electrodes, palladium electrodes as reference electrodes, and carbon auxiliary electrodes. The detector and the column were thermostated. Temperatures under 30 $^{\circ}\text{C}$ were obtained using an air-conditioner (ET9, Italy). The sample (5 μl) was injected manually using a glass syringe (Hamilton, USA).

High-performance liquid chromatography coupled with diode array detection

An HP 1100 liquid chromatographic system (Hewlett Packard, Waldbronn, Germany) was equipped with a vacuum degasser (G1322A), a binary pump (G1312A), an autosampler (G1313A), a column thermostat (G1316A), and a UV-VIS diode array detector (model G1315A) working at 190–690 nm. ChemStation software (Rev. A 08.01) controlled the whole liquid chromatographic system. Spectra were recorded in the range of 190–400 nm (SBW 100 nm). Biotin was separated on an Atlantis dC18 reversed-phase chromatographic column (150 \times 2.1 mm, 3 μm particle size, Waters Corp. Milford, USA) in an isocratic mode with methanol–0.01% trifluoroacetic acid in Milli-Q water (35:65). The flow rate was 1.5 ml min^{-1} . The temperature of the column and detector was set to 35 $^{\circ}\text{C}$. The autosampler injection volume was 5 μl .

Avidin maize sample and its preparation

The construction of plasmids used to transform the maize with the chicken avidin gene, the transformation itself, tissue culture, and the generation of avidin-expressing transgenic plants were all performed as

described previously [50]. Briefly, 10 g of transgenic or non-transgenic (control) maize kernels were ground for 1 min in a coffee grinder. The resulting powder was extracted for 1 h at 4 $^{\circ}\text{C}$ with constant stirring in 50 ml of a buffer containing 50 mM sodium carbonate (pH 11.0), 500 mM NaCl, 5 mM EDTA, and 0.05% (v/v) Tween-20. The extraction mixture was centrifuged at 16,000g for 15 min (Jouan MR 23i) at 4 $^{\circ}\text{C}$. The supernatant was removed and filtered through four layers of cheesecloth. The filtrate was then centrifuged at 14,500g for 15 min at 4 $^{\circ}\text{C}$. The supernatant was recovered and the pH adjusted to 10.5, and then centrifuged at 14,000g for 30 min (Eppendorf 5402) at 4 $^{\circ}\text{C}$ [14, 50]. The final supernatant was recovered and subsequently used to prepare the modified CPE.

Extraction procedures for the pharmaceutical drug

Pharmaceutical drug used

Tablets of the pharmaceutical drug Biotin (Nature's Bounty, Inc., USA) were used. An Ika A11 basic grinder (IKA Werke GmbH and Co., Staufen, KG, Germany) was used for homogenization of the tablets.

Accelerated solvent extraction procedure

An accelerated solvent extraction procedure [30, 51] was performed in a PSE (Applied Separations, USA) pressurized solvent extractor. The homogenized (0.500 \pm 0.005 g) pharmaceutical drug Biotin was placed in a small filter paper envelope and sprinkled with 3.0 g SPE-ed matrix, 1.5 g florisisl (15.5% MgO, 84% SO₂, 0.5% Na₂SO₄; pH 8.5; 60–100 μm particle size) and 3.0 g Ottawa sand (Allentown, PA, USA). Flavone was used as an internal standard and was pipetted onto the top of the sample. The mixture was placed into a 10 ml stainless steel extraction cell and extracted under controlled conditions in one step: pre-heating period (5 min), the solvent was 50% methanol; temperature 100 $^{\circ}\text{C}$, pressure 150 bar; two extraction cycles (5 min), 90 s using pressurized nitrogen. The final extracts were collected in 40 ml glass vials. The obtained extract was filtered through a 0.45 μm Teflon membrane filter (MetaChem, Torrance, CA, USA) prior to injection into the HPLC system.

Water extraction

Sonication of the homogenised sample (0.500 \pm 0.005 g) was performed at laboratory temperature for 15 min on a K5 Sonicator (Slovakia) at 38 kHz and 150 W. Water (10 ml) was used as an extraction solvent. Flavone was used as an internal standard and was pipetted onto the top of the sample before sonication. The obtained extract was filtered through a Teflon disc filter (0.45 μm , 13 mm diameter, Alltech Associates, Deerfield, IL, USA) prior to injection into the HPLC system.

Phosphoric acid extraction

The homogenization of the pharmaceutical drug sample (0.500 ± 0.005 g) was performed at laboratory temperature for 5 min with stirring at 250 rpm. The extraction solvent (10 ml) was an aqueous solution of phosphoric acid at a final concentration of 1.5% (v/v) [6]. The sample was sonicated for 15 min. Flavone was used as an internal standard and was pipetted onto the top of the sample before stirring. The obtained extract was filtered through a Teflon disc filter ($0.45 \mu\text{m}$, 13 mm diameter, Alltech Associates, Deerfield, IL, USA) prior to injection into the HPLC system.

Accuracy, precision and recovery

Accuracy, precision and recovery of the biotin were evaluated with homogenates (pharmaceutical drug) spiked with standards. Before an extraction, $100 \mu\text{l}$ of biotin standard (various concentrations from 10 ng ml^{-1} to 100 ng ml^{-1}), $100 \mu\text{l}$ water and $100 \mu\text{l}$ flavone were added to the pharmaceutical sample. The precision (coefficient of variation; %CV) of the intra-day assay was measured in six homogenates. Inter-day precision was determined by analyzing six homogenates over a five-day period. Homogenates were assayed blindly and biotin concentrations were derived from the calibration curves. Accuracy was evaluated by comparing the estimated concentration with the known concentrations of biotin. Calculation of accuracy (%Bias), precision (%CV) and recovery was expressed as described in [52, 53].

Statistical analysis

STATGRAPHICS software (Statistical Graphics Corp, USA) were used for statistical analyses. Results are expressed as mean \pm SD unless stated otherwise. A value of $p < 0.05$ was considered significant.

Results and discussion

Adsorptive transfer stripping voltammetry of avidin at CPE

Avidin is an important protein that contains a variety of amino acids in its structure (Fig. 1A). From an electrochemical point of view, only tyrosine (Y) and tryptophan (W) have been found to be electroactive using a variety of electrodes [54, 55]. Square-wave voltammetric analysis at solid carbon electrodes is not very sensitive and yields only poorly developed signals. However, by using a CPE and sophisticated baseline correction, we obtained well-defined voltammetric signals for both Y and W at 0.78 and 0.92 V vs. Ag/AgCl/3 M KCl, respectively (Fig. 1C). The peaks were obtained at

$100 \mu\text{g ml}^{-1}$ ($\sim 7 \mu\text{M}$) of avidin by using the adsorptive transfer stripping square-wave voltammetry technique. A scheme showing the transfer technique is provided in Fig. 1B. The technique is based on the strong adsorption of avidin on the electrode surface and subsequent electrode transfer to a washing solution and then to an electrolyte that does not contain any avidin in bulk solution, where electrochemical measurements are performed. An advantage of using this approach is that only a small amount of sample is required (6 and/or 3 μl drops) per voltammetric analysis. These electrochemical transfer techniques have been previously described in detail in [56–58].

We determined that the peak height was linearly dependent on the time of avidin accumulation up to a time of approximately 2 min, after which the height decreased (Fig. 1D). It should be noted that our measurements were performed without any stirring; when the solution is stirred, the same coverage saturation should be reached in a shorter accumulation time under the same experimental conditions. To determine the dependence of peak height on avidin concentration, we varied the avidin concentration from 0 to $100 \mu\text{g ml}^{-1}$. The dependence was found to be linear over the studied concentration range, with $R^2 = 0.9978$ (Fig. 1E). The limit of detection ($3 \times S/N$ ratio criterion) was about 500 pg ml^{-1} ($\sim 3 \text{ fM}$). The reproducibility of the method was checked ($n = 5$) by performing determinations of 6 and 3 μl drops of avidin ($1 \mu\text{g ml}^{-1}$) at an accumulation time of 120 s, with the relative standard deviations (RSD) found to be only 6.9 and 9.8%, respectively.

Electrochemical behavior of avidin in modified CPE

Avidin in CPE

Avidin-modified CPE was prepared by simply mixing a desired quantity of avidin into a conventionally-prepared CPE composed of graphite powder and mineral oil (Fig. 2A). Compared to CPE, avidin-modified CPE exhibited similar voltammetric signals for Y and W (for comparison see Figs. 1C and 2A). When the amount of carbon powder (at constant concentration of avidin of $10 \mu\text{g ml}^{-1}$) was changed in the paste mixture, we observed the highest voltammetric signal at a powder/oil ratio of 70/30 (w/w) (Fig. 2B). This result is in good agreement with other studies of unmodified CPE [14, 40, 59] and indicates that the avidin-modified CPE possesses electrochemical properties similar to those of the CPE. We also investigated whether avidin was washed out from the paste by keeping the modified electrode in the electrolyte solution. The voltammetric signal decreased by about 15% over an incubation time of 20 min (Fig. 2C). From these results, it is clear that an accumulation time of just 2 min would not markedly affect the peak height of avidin.

Electrochemical behaviour of avidin on CPE surface

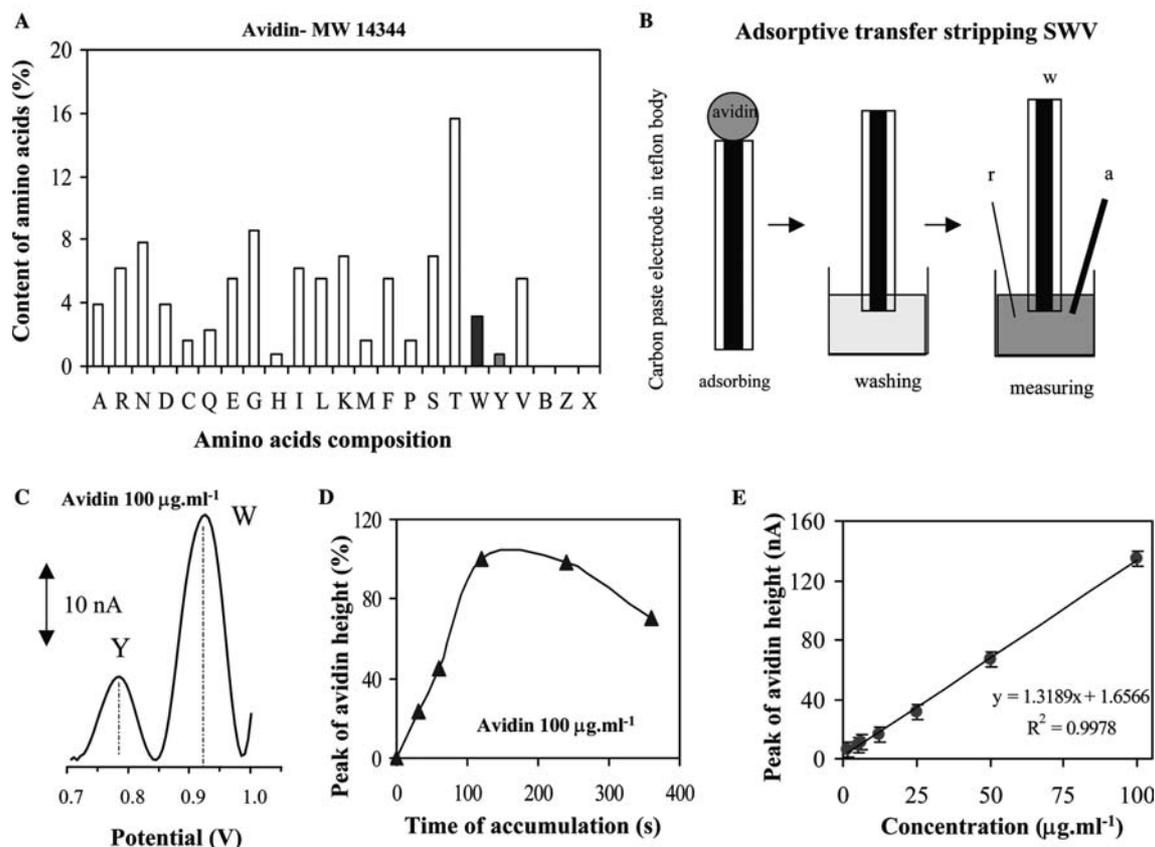


Fig. 1 A Amino acid composition of avidin; MW: 14,344 Da. **B** Adsorptive transfer stripping (AdTS) technique used with the carbon paste electrode (CPE). **C** Voltammogram of avidin ($100 \mu\text{g ml}^{-1}$; $t_A = 120 \text{ s}$) obtained by CPE. The voltammetric signal corresponds to the oxidation of Trp (Y) and Tyr (W) residues in the avidin molecule. **D** Dependence of AdTS SWV peak height of avidin on time of accumulation. **E** Dependence of AdTS SWV avidin peak height on avidin concentration. All measurements were performed in acetate buffer (pH 4). The SWV method was used with the following parameters: initial potential = 0.1 V, end potential = 1.3 V, step potential = 5 mV, amplitude = 25 mV, frequency = 200 Hz

As expected, the voltammetric signal was dependent on the amount of avidin present in the avidin-modified CPE. A linear increase in peak height was observed as the concentration of avidin in the CPE increased up to an avidin concentration of $500 \mu\text{g ml}^{-1}$, when a fresh CPE surface was prepared for each measurement (RSD = 3.4%, $n = 3$). The calibration curve for the concentration range examined was linear, with a regression line of $y = 1.00x + 9.04$ ($R^2 = 0.996$) (Fig. 1D). The lowest concentration at which the voltammetric signal was observable under our experimental conditions was 10 ng ml^{-1} of avidin in the avidin-modified CPE (700 fM ; RSD = 3.4%, $n = 3$). We assume that the different avidin detection limits obtained by CPE versus CMPE might be due to different arrangements of the analyte in active electrode surface-adsorption versus incorporation.

Determination of avidin in transgenic avidin maize extract using avidin extract-modified CPE

Modern genetic engineering technologies make it possible to prepare plant species that produce a number of value-added proteins (such as monoclonal antibodies, antigens for vaccines, and so on) [31, 50, 60–64]. Transgenic maize that produces avidin as an insecticidal agent against insect pests, was recently prepared [31, 32, 50]. In our previous work [14], we mixed the plant seed extract obtained from transgenic maize with carbon powder. Then we prepared the avidin extract-modified CPE. The height of the resulting electrochemical signal corresponded to the concentration of avidin present in the maize plant sample. In the present study, we determined how the avidin peak height changes depending on the amount of maize plant tissue extract added to the carbon powder (each avidin-modified CPE was used only for one measurement; RSD = 7.8%, $n = 3$). After the plant seed homogenization step, the CPE was modified by the plant extract and the voltammetric response was recorded. We were able to observe well-developed square-wave voltammetric signals in all tested samples (inset in Fig. 2E).

A relatively easy, rapid and easily applicable electrochemical procedure for avidin detection in transgenic seeds was developed. For analytical purposes we constructed a calibration curve showing a linear relationship between the avidin concentration and the volume of a

Electrochemical behaviour of avidin in carbon paste

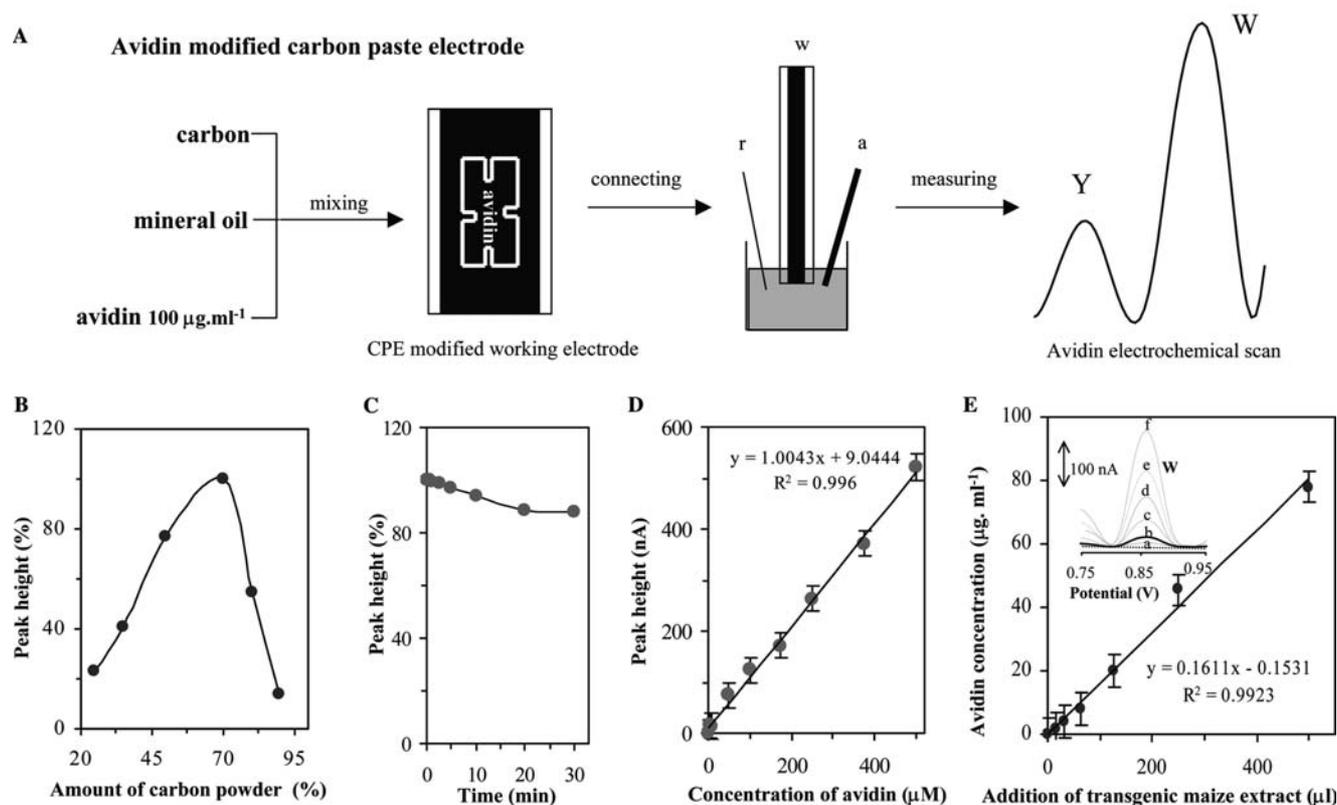


Fig. 2 Electrochemical behavior of the avidin-modified CPE. **A** Scheme of avidin-modified CPE: mixing, connection and resulting SW voltammogram of avidin. **B** Influence of amount of carbon powder in the carbon paste. **C** Dependence of avidin-modified CPE SWV peak height on time that the electrode was immersed in the background electrolyte. **D** Dependence of AdTS SWV avidin peak height on avidin concentration (avidin in CPE). **E** Dependence of AdTS SWV avidin peak height on avidin concentration in transgenic maize extract (inset: voltammograms of transgenic maize extract in modified CPE at different amounts of extract in CPE: a, 15.6; b, 31.3; c, 62.5; d, 125; e, 250; f, 500 μl ; dotted line is background electrolyte). A peak height of 100% represents 100 $\mu\text{g ml}^{-1}$ avidin concentration in avidin-modified CPE. Other experimental conditions are the same as in Fig. 1

transgenic maize extract ($y = 0.1611x - 0.1531$; $R^2 = 0.9923$) (Fig. 2E). The peak height of the voltammetric signal increased linearly with as the amount of transgenic seed extract added into the avidin-modified CPE was increased from 15.6 μl to 500 μl . The lowest detectable volume of the plant extract was about 15 μl , which corresponds to 2.5 $\mu\text{g ml}^{-1}$ of avidin in the sample. When we tested a non-transgenic avidin plant, only a very small signal was observed (curve *a* in Fig. 2E, inset). To compare the avidin signal from the transgenic seeds to that from the non-transgenic ones, we prepared a set of extract samples from the commercial maize food (farina, grits, and grout). Voltammetric signals obtained from these samples were very low and their heights were about 2–8% relative to the signal obtained from the transgenic sample extract (data not shown). These results were in good agreement with our previously

published results [14]. This procedure can be used to analyse other transgenic products that contain avidin, such as avidin apple, tobacco and rice [33, 65, 66]

Avidin-modified CPE in the presence of biotin

Study of the interaction between avidin and biotin

The interaction between avidin and biotin is one of the strongest known associations between a protein and its ligand [10, 67]. Because of the extremely strong interaction of this complex, avidin–biotin technology is used in various experimental techniques, including electrochemical ones [21–25, 35, 68, 69]. To evaluate the properties of an avidin-modified electrode in the presence of biotin, we added biotin to the background electrolyte and followed the voltammetric response of avidin. We kept the amount of avidin in the avidin-modified CPE and the accumulation time constant (10 $\mu\text{g ml}^{-1}$; 120 s) and changed the concentration of biotin in the electrochemical cell from 0.5 μM to 14 μM . We observed that the voltammetric signal of avidin decreased with increasing concentration of biotin. The observed decrease in avidin signal after addition of biotin is probably caused by the fact that the avidin–biotin complex formed could mask some of the electroactive tryptophans and tyrosine of avidin. The electrochemical signal of avidin was nearly undetectable at 10 μM biotin (Fig. 3A:a). The shape of the response

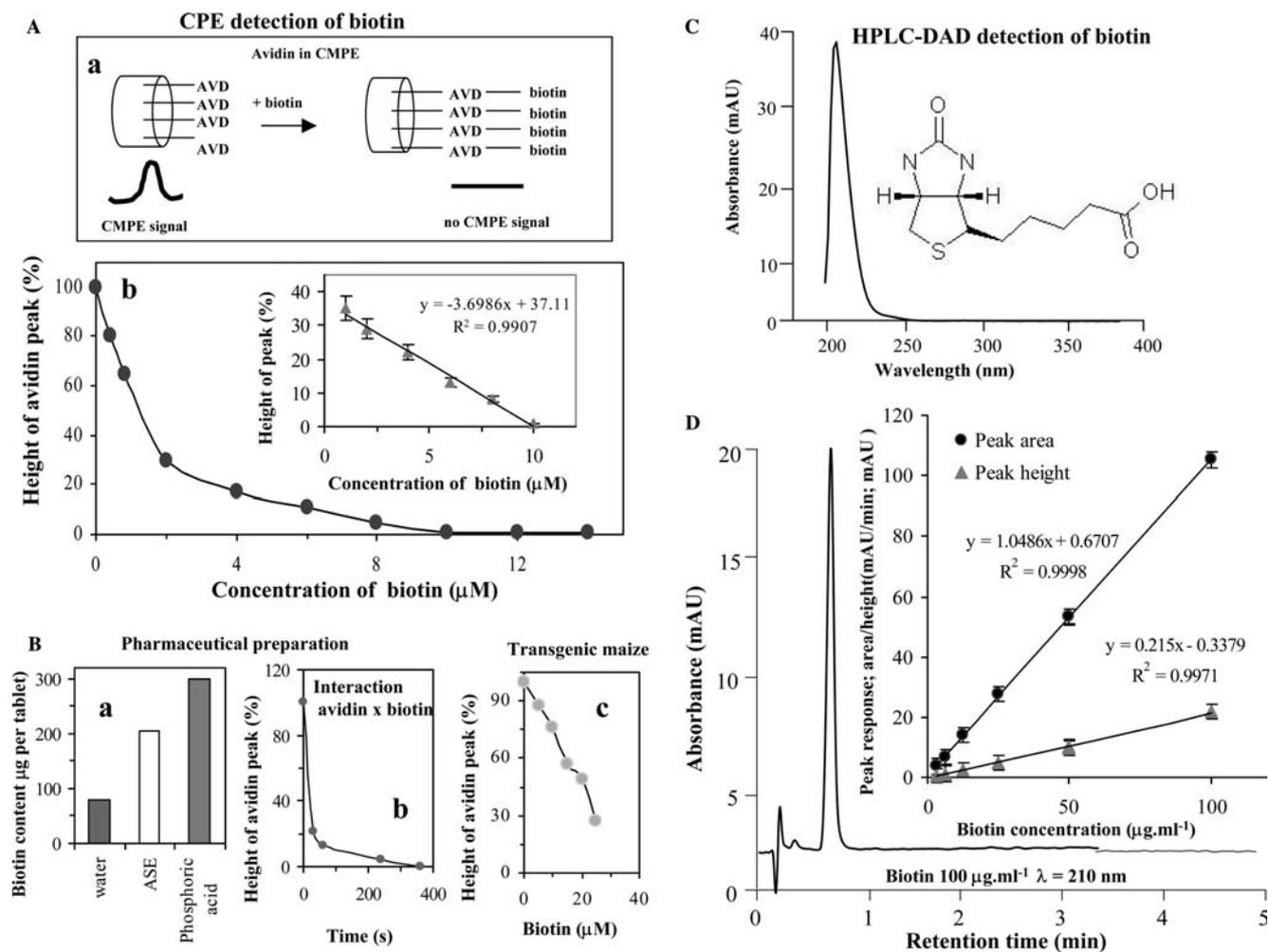


Fig. 3 Interaction of avidin contained in avidin-modified CPE with biotin. **A** (a) Scheme of avidin-modified CPE interaction with biotin present in solution and resulting voltammetric signal. (b) Dependence of AdTS SWV avidin peak height on concentration of biotin in solution; inset: the same dependence for 2–10 μM concentration of biotin in solution. **B** Avidin-modified CPE determination of biotin in a pharmaceutical preparation. (a) The pharmaceutical samples containing biotin were prepared by three different extraction procedures (for other details, see the “Experimental” section and Table 1). 100% of biotin represents $300 \mu\text{g g}^{-1}$ in one tablet. (b) Dependence of AdTS SWV avidin peak height on time of interaction with biotin. (c) Dependence of AdTS SWV avidin peak height (CPE electrode modified by plant seed extract obtained from transgenic maize) on biotin concentration. Peak height of 100% represents $100 \mu\text{g ml}^{-1}$ avidin in avidin-modified CPE. Other experimental conditions were the same as in Fig. 1. **C** HPLC diode array (HPLC-DAD) spectrophotometric measurements of biotin. Chemical structure and UV spectrum of biotin. **D** HPLC-DAD chromatogram of biotin ($100 \mu\text{g ml}^{-1}$); inset: dependence of peak height and/or area on biotin concentration. Detection wavelength was 210 nm. For other chromatographic conditions, see the “Experimental” section

curve was exponential ($y = 80.2 e^{-0.36x}$). For analytical purposes we attempted to divide the concentration interval into two parts in order to be able to apply a titration curve. The first part (concentration of biotin = 0–2 μM) was strictly linear ($y = -42.73x + 99.92$;

$R^2 = 1$). The second part (concentration of biotin = 2–10 μM) had the following parameters: $y = -3.70x + 37.11$; $R^2 = 0.9907$; $n = 5$; $\text{RSD} = 5.9\%$ (see in inset Fig. 3A,B).

Detection of biotin by avidin-modified CPE

Biotin is also known as vitamin H (Fig. 3) [1]. The determination of biotin in pharmaceutical preparations and/or food samples is relatively difficult. The most commonly used biotin detection methods are based on using high performance liquid chromatography with UV detection or on using microbiological techniques [5, 20]. We tested the SWV voltammetric method for avidin detection by indirectly determining the biotin in a pharmaceutical drug. The preparation of the sample was a very important factor for accurate and precise analysis. In the case of biotin detection, we selected three different extraction procedures: water extraction, accelerated solvent extraction (ASE) and extraction using phosphoric acid (for additional details, see the “Experimental” section) (Fig. 3B:a). Recoveries and amounts of biotin extracted by these procedures are given in Tables 1 and 2. We selected the extraction by phos-

Table 1 Biotin detected in a pharmaceutical drug using three procedures based upon the use of avidin-modified CPE or HPLC-ED

	Avidin-modified CPE	HPLC –DAD
Extraction procedure ^c	Found μg per tablet ^{a,b}	Found μg per tablet ^{a,b}
Water	80 \pm 4	76 \pm 3
ASE	205 \pm 6	209 \pm 5
Phosphoric acid	299 \pm 4	303 \pm 7

^aDeclared biotin amount in one tablet is 300 μg

^bAmount expressed as mean \pm SD ($n=6$)

^cExtraction procedures are described in the “Experimental” section

phoric acid as the best extraction procedure since it gave the highest amount of extracted biotin. The reproducibility of the procedure was determined using six repetitive analyses of representative samples over five days (Table 3). The biotin amounts measured were in good agreement with the declared values.

Electrochemical indirect determination of biotin using the avidin-modified CPE ($10 \mu\text{g ml}^{-1}$) was performed at an avidin–biotin interaction time of 120 s with constant stirring (1,430 rpm). We observed that the electrochemical signal of avidin rapidly decreased after the avidin–biotin interaction occurred at the CPE surface (Fig. 3B:b). In addition we obtained similar results with the interaction between biotin and a plant avidin extract-modified CPE (Fig. 3B:c). This simple testing method can be used for rapid, low-cost and selective determinations of avidin in plants and other types of materials.

Direct detection of biotin by HPLC with diode array detection

The most common method used for the direct determination of biotin is high performance liquid chromatography (HPLC) with diode array detection or fluorescence detection [1, 4–6, 20, 70]. On the basis of our determinations of biological compounds by high performance liquid chromatography with diode array detection (HPLC-DAD) published previously [70], we selected the same analytical method for the detection of biotin. The UV spectrum of biotin presented in Fig. 3C

clearly shows that, in order to obtain a high signal-to-noise ratio, the detection should be performed using a wavelength that is as low as is possible. Hence, the detection was performed at $\lambda=210 \text{ nm}$ during the HPLC experiments. Detection at wavelengths below $\lambda=210 \text{ nm}$ resulted in background noise that was too high due to the presence of methanol in the mobile phase (cut-off at $\lambda=200 \text{ nm}$). A typical HPLC-DAD chromatogram of biotin ($100 \mu\text{g ml}^{-1}$) is shown in Fig. 3D. The dependence of peak area and/or height on biotin concentration is shown in the inset to Fig. 3D; the equations for the obtained bisectors were $y=1.0486x+0.6707$; $R^2=0.9998$ for peak area and $y=0.2150x-0.3379$; $R^2=0.9971$ for peak height, respectively ($n=5$; $\text{SD}=2.5\%$). For direct HPLC-DAD biotin detection in a pharmaceutical drug, we tested three different extraction procedures as described above. The results are summarized in Tables 1 and 2.

Determination of avidin by FIA with electrochemical detection

On the basis of the above-mentioned results obtained via electrochemical detection of avidin and/or biotin, we also wanted to know whether we would be able to detect avidin and/or biotin by flow injection analysis with electrochemical detection. The electrochemical detector included two low-volume flow-through analytical cells containing eight carbon porous working electrodes [71]. Each carbon porous working electrode was connected to two reference and two counter electrodes (Fig. 4Aa).

Influence of the applied potentials of the working electrodes of the CoulArray detector

Next, the optimal potentials of the CoulArray detector's working electrodes were identified so that we could maximize the sensitivity of the determination of avidin. The flow injection analysis conditions utilized were an isocratic mobile phase consisting of 94% 0.02 M phosphate buffer (pH 6.7; solvent A) and 6% acetonitrile (solvent B). The flow rate of the mobile phase was 0.5 ml min^{-1} . The column and detector temperatures were both $25 \text{ }^\circ\text{C}$. Potentials of 600, 650, 700, 750, 800,

Table 2 Recovery of biotin from pharmaceutical drug homogenate using modified CPE or HPLC-DAD methods in triplicate ($n=6$)

Detection method	Extraction procedure	Homogenate ($\mu\text{g ml}^{-1}$) ^{a,b}	Spiking biotin ($\mu\text{g ml}^{-1}$) ^{a,b}	Homogenate + spiked biotin ($\mu\text{g ml}^{-1}$) ^{a,b}	Recovery (%)
Modified CPE	Water	5.5 \pm 0.2 (3.6)	5.8 \pm 0.2 (3.4)	11.8 \pm 0.6 (5.1)	104
	ASE	5.6 \pm 0.3 (5.4)	5.4 \pm 0.4 (7.4)	10.6 \pm 0.5 (4.7)	96
	Phosphoric acid	5.1 \pm 0.3 (5.9)	5.5 \pm 0.3 (5.5)	10.9 \pm 0.5 (4.6)	103
HPLC-DAD	Water	5.5 \pm 0.4 (7.3)	5.2 \pm 0.2 (3.8)	11.2 \pm 0.6 (5.4)	105
	ASE	5.3 \pm 0.1 (1.9)	5.3 \pm 0.4 (7.5)	11.0 \pm 0.4 (3.6)	104
	Phosphoric acid	5.4 \pm 0.1 (1.9)	5.1 \pm 0.1 (2.0)	10.3 \pm 0.3 (2.9)	98

^aBiotin amounts are per one milliliter of diluted solution obtained by extraction

^bResults expressed as mean \pm SD (CV%) ($n=6$)

Table 3 Precision and recovery of biotin for pharmaceutical drug analysis ($n=6$)

Detection method	Extraction procedure	Homogenate ($\mu\text{g ml}^{-1}$) ^{a,b}	Spiking biotin ($\mu\text{g ml}^{-1}$) ^{a,b}	Homogenate + spiked biotin ($\mu\text{g ml}^{-1}$) ^{a,b}	Recovery (%)
Modified CPE	Intra-day ($n=6$)	5.2 ± 0.2 (3.8)	5.7 ± 0.3 (5.3)	10.8 ± 0.3 (2.8)	99
	Inter-day ($n=30$)	5.0 ± 0.4 (8.0)	5.1 ± 0.3 (5.9)	10.4 ± 0.8 (7.7)	103
HPLC-DAD	Intra-day ($n=6$)	5.3 ± 0.1 (1.9)	5.4 ± 0.4 (7.4)	11.0 ± 0.5 (4.5)	103
	Inter-day ($n=30$)	5.5 ± 0.5 (9.1)	5.3 ± 0.4 (7.5)	10.5 ± 0.9 (8.6)	97

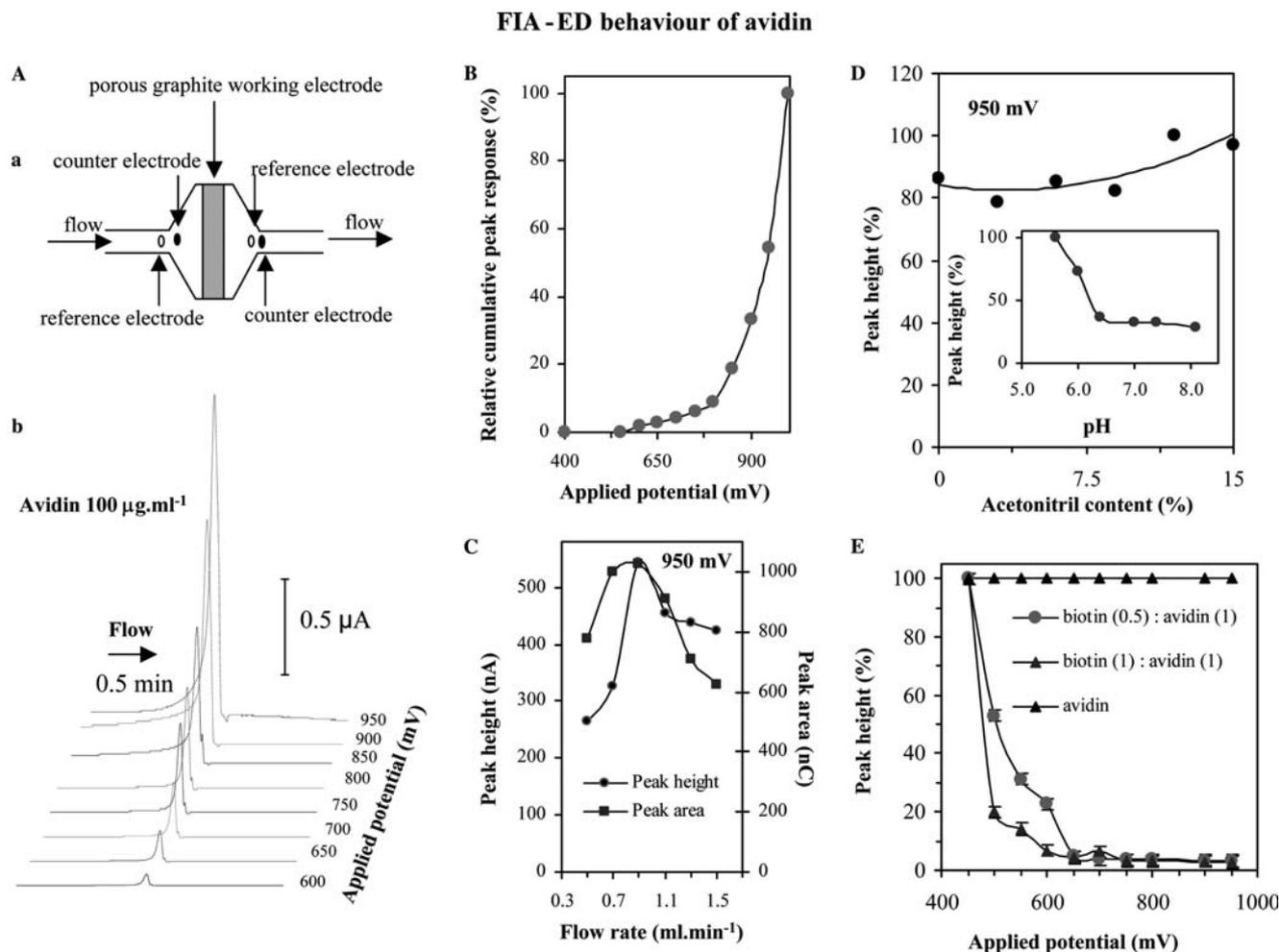
^aBiotin amounts per one milliliter of diluted solution obtained by the phosphoric acid extraction method

^bResults expressed as mean \pm SD (CV%)

850, 900 and 950 mV were applied to the porous graphite working electrodes. According to the results obtained from using the electrochemical detection

method (Figs. 1 and 2), we expected the highest electrochemical response from avidin to occur at 900 mV. The full scan of the FIA-ED detection of avidin is shown in Fig. 4A:b. The resulting hydrodynamic voltammogram (the dependence of current response on the cell detector applied potential) is shown in Fig. 4B. A potential obtained from the limit diffusion current area or from the point of the highest current difference and the smallest potential difference is the most suitable for electrochemically detecting the studied substances [72]. The most effective potential (950 mV) was selected from the point of the highest current difference and the smallest potential difference for measurements of avidin (Fig. 4B).

Fig. 4 Analysis of avidin by flow injection analysis with electrochemical detection (FIA-ED). **A** (a) Scheme of the detection cell with the graphite porous working electrode; (b) Full scans at different applied potentials. **B** Hydrodynamic voltammogram of avidin ($100 \mu\text{g ml}^{-1}$) at applied potentials from 400 mV to 1,000 mV. **C** Influence of the flow rate of the mobile phase. **D** Acetonitrile content in the mobile phase; inset: effect of pH on peak height of avidin. **E** Study of the avidin–biotin interaction by FIA-ED. Peak height of 100% represents $100 \mu\text{g ml}^{-1}$ avidin concentration. FIA-ED conditions: a mobile phase containing 0.02 M phosphate buffer and acetonitrile was used; column and detector temperature = 25 °C



Influence of mobile phase flow rate

The flow rate of the mobile phase is very important for chromatographic analysis because of its significant influence on the retention time, height and character of the analytical signals from the detected compounds [73]. The effect of different flow rates (0.3, 0.6, 0.9, 1.2 and 1.5 ml min⁻¹) on the avidin peak height was studied (Fig. 4C). According to the height of the electrochemical avidin signals obtained, the most suitable flow rate of the isocratic mobile phase consisting of 94% 0.02 M phosphate buffer (pH 6.7) and 6% acetonitrile was 0.9 ml min⁻¹. The current response of the avidin decreased by up to 85–90% at the highest selected mobile phase flow rates (1.2 and 1.5 ml min⁻¹) in comparison with the current response of avidin obtained at a flow rate of 0.9 ml min⁻¹. This phenomenon is probably caused by a shorter preconcentration time of the solute on the working electrode's surface (Fig. 4C).

Influence of acetonitrile levels and pH of phosphate buffer in the HPLC mobile phase

The organic component of the mobile phase can negatively influence the electrochemical response of a sample [70, 72, 74, 75]. In our experiments, we used the organic solvent of the mobile phase to facilitate chromatographic separation. The levels of acetonitrile (0–15% ACN) used in the mobile phase did not markedly influence the height of the avidin signal (Fig. 4A). The highest response was obtained at 15% acetonitrile in the mobile phase. Inorganic components of the mobile phase can also significantly influence the detection of avidin [70, 72, 74, 75]. We studied the influence of the pH of the phosphate buffer in the range 5.6–8.2. The highest electrochemical signal was obtained at the lowest pH value tested, pH 5.6 (see inset of Fig. 4D).

The most effective FIA-ED parameters

The best FIA-ED detection conditions for avidin were as follows: isocratic mobile phase, 0.02 M phosphate buffer (pH 5.6):acetonitrile in a ratio of 85:15; flow rate,

0.9 ml min⁻¹; column and detector temperature, 25 °C; electrode potential, 950 mV.

Influence of avidin concentration on its electrochemical response

We determined the detection limit of avidin by FIA-ED using the optimized method parameters. The limit was 392 fmol of avidin per injection (5 µl) for a signal-to-noise ratio S/N=3. The RSD was only about 3.9% ($n=7$).

Study of avidin-biotin interaction by FIA-ED

There are only a few published methods that are used for biotin detection. Direct electrochemical determination of biotin by FIA-ED with a silver electrode was described in 1986 [7]. We also determined biotin using an optimized FIA-ED technique. The results obtained will be published elsewhere. Besides direct FIA-ED determination of biotin and/or avidin, we used the optimized HPLC-ED technique to study the avidin-biotin interaction at full scan. Before injection of the avidin and biotin into the FIA-ED system, the biotin was mixed with avidin in two molar ratios (avidin: biotin; 1:0.5 and 1:1). It clearly follows from the results shown in Fig. 4E that the electrochemical signal of avidin decreased with increasing biotin concentration. During the electrochemical study of the avidin-biotin interaction we did not observe any signal from biotin, because all molecules of biotin were associated with avidin.

Conclusions

We constructed a protein-modified voltammetric bioelectrode by incorporating a protein into a CPE (avidin-modified CPE), which allows specific analysis of proteins of interest as well as their ligands. This technology offers useful and promising possibilities in the field of biosensor development. Square-wave voltammetry scans oxidative signals generated from Trp and Tyr residues in the proteins. We also demonstrated the application of

Table 4 Limits of detection (LODs for S/N=3) for avidin and biotin using four analytical methods

Detected compounds	Detection procedure											
	AdTS SWV CPE			SWV Modified CPE			HPLC-UV			FIA-ED		
	pg	amol	fM	ng	fmol	fM	pg	pmol	pM	ng	fmol	pM
Avidin	3 or 1.5 ^a	200 or 100 ^a	3	2.5 ^b	174 ^b	700	ND	ND	ND	5.6 ^c	390 ^c	78
Biotin	ND	ND	ND	1.8	7.6×10 ³	2.5×10 ³	750	3.0	614	8.1 ^c	33.5×10 ^{3c}	6.6×10 ³

ND not detected

^aDetection limits per drop (6 or 3 µl)

^bDetection limits per 250 µl of modified carbon paste

^cDetection limits per injection (5 µl)

this pulse voltammetric method for detection of avidin extracted from transgenic maize. In the case of biotin determination, we applied this voltammetric method in conjunction with high performance liquid chromatography with electrochemical detection. The results obtained demonstrate that the method is very sensitive and specific. The limits of detection for the avidin and/or biotin are summarized in Table 4.

Acknowledgements This work was supported by grants: GA CR No. 525/04/P132, No. 203/02/0422, MSMT No. LN 00A081, GAAV No. A1163201 and IGA MZLU No. 3/2004.

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