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Development of immunosensors based on optical waveguide lightmode spectroscopy (OWLS) technique for determining active substance in herbs

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ABSTRACT

For the quick and reliable quantification of special active substances derived from herbs, a new type of immunosensor based on optical waveguide lightmode spectroscopy (OWLS) detection was investigated. Artemisinin, an antimalarial drug derived from the sweet wormwood plant *Artemisia annua* is a sesquiterpene lactone endoperoxide, and it is distilled from the dried leaves or flower clusters of *A. annua*. Numerous derivatives of artemisinin, including artesunate and artemether, are also being used in the treatment of malaria, and these compounds have recently gained utility also as anticancer agents. To quantitatively determine the presence of these biologically effective substances in various herbs, a novel OWLS-based immunosensor was investigated. To create regenerable sensitized surfaces in the OWLS technique so that the sensor can be applied several times, the antigen or the antibody were immobilized by covalent attachment to the silanized surfaces of the OWLS chips. When measuring with the antibody capture mode antibodies raised against the appropriate artemisinin derivative (artemisinin, artesunate or artemether) were immobilized on the sensor surface and the linear measuring range was determined. During the antigen capture measurement the protein conjugate of the analyte was immobilized on the waveguide surface. Standard solutions containing different amounts of the appropriate standard were mixed with antibodies at optimized concentration, the mixture was incubated and injected into the flow-injection analysis system of OWLS. Binding of the antibodies in the sample to the coated surface is competed for with free antigen in the sample, and only antibodies that remained in free form in the mixture bound to immobilized antigen-conjugates. The amount of antibodies bound to the surface of the chip was inversely proportional to the active substance content in the samples. The dynamic measuring range and the relative substrate specificity of the serum on artemisinin and its derivatives were studied. Herbs of different origin and herbal supplement products were analyzed using the newly developed method and a correlation was studied with HPLC/DAD/MS reference method.

1. Introduction

Environmental and food safety as a key aspect in food production and processing has come into the forefront during the last decades. Food supplements used for dietary, nutritional or medicinal purposes, characterized by high content of vitamins, minerals or other substances with nutritional or physiological effects, represent an important segment in the food sector. Some of these dietary supplements are being advised to affect the spread, as well as morbidity and mortality of infectious diseases e.g. malaria [1,2].

The composition of food supplements and dietetic products, including botanicals, seems to be of substantial awareness for food control authorities, as certain, in some cases undeclared components found in these products raise human health concerns. Due

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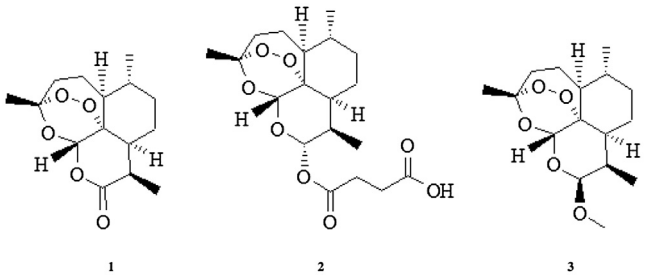

Fig. 1. The chemical structure of artemisinin (1), artesunate (2) and artemether (3).

to the efficient action of the Rapid Alert System for Food and Feed (RASFF) of the EU, numerous alerting or warning signals have been issued during recent years regarding the active substance levels or the presence of unauthorized compounds originated from herb infusions or herbal supplement products. Summarizing the data published by RASFF, the number of notifications between 2005 and 2014 increased ten-fold for food supplements, with the reason of the notification being the presence of unauthorized substances (50%), unauthorized novel food ingredients (16%), unauthorized products (13%), unauthorized placing on the market (11%) or too high content of the substance in question (9%) [3].

The herb *Artemisia annua* has been used for many centuries in Chinese traditional medicine as a treatment for fever and malaria [4]. In the early seventies Tu [5] isolated a colorless, crystalline substance from the leafy portions of the plant. This compound, called qinghaosu (artemisinin), is an endoperoxide sesquiterpene lactone (Fig. 1). Derivatives of artemisinin, such as dihydroartemisinin, artemether, and the water-soluble sodium salt of artesunate (Fig. 1) have the same effect that appears to be more efficient, than artemisinin itself [6]. Lately, according to the results of early cancer treatment research studies, artemisinin has become a promising active substance in cancer therapy, therefore several food supplements and herb extracts containing artemisinin has appeared on the market. The determination of the concentration of the biologically active compound of these products is of key importance.

The current analytical methods used for this task are high performance liquid chromatography (HPLC) and coupled techniques [7]. Mannan et al. [8] investigated a quantitative comparison of artemisinin concentration in different *Artemisia* species and plant parts by using HPLC-DAD method (260 nm). The most effective method for the determination of artemisinin and its derivatives is chromatography coupled with mass spectrometry (HPLC–MS) [9,10] and the analysis based on HPLC/MS/MS [11–13]. Koesdjojo [14] developed a rapid, inexpensive and simple kit for the detection of the presence of the active ingredient in different antimalarial drugs on the basis of paper microfluidics with a semi-quantitative colorimetric assay specific to artesunate in the range of 0.0–20 mg mL^{-1}.

Beside the intensively studied HPLC techniques, different immunoanalytical methods have also been used in the last decades for quick monitoring of the biologically active compounds. Eggelte [15] reported the production of monoclonal antibodies against artelinic acid; its specificity on artemisinin and its metabolites, and their application in an inhibition ELISA test for urine samples. Different research groups developed indirect competitive enzyme linked immunosorbent assays (icELISA) by using novel monoclonal and recombinant antibodies. The cross-reactivity (CR) of the different compounds and the working range of the icELISA methods were defined in each study [6,16–18].

Biosensors – especially adaptation of nanotechnology – represent a cutting-edge frontier in environmental and food analysis, so several biosensors are reported based on highly selective and sensitive measuring methods applying different means of detection. Reys [19] presented an amperometric sensor for the analysis of artemisinin in neutral medium based on electrocatalytic reduction of artemisinin by the catalytic activity of hemin immobilized carbon paste electrode modified by silica at a potential of −0.5 V vs. Ag/AgCl, in a linear response range from 50 to 1000 nmol L^{-1}.

Wu and co-workers [20] studied the use of highly selective molecularly imprinted membranes (MIMs) for artemisinin on poly(vinylidene fluoride) micro-filtration membrane. Gong and Cao [21] investigated molecularly imprinted polymers (MIPs) for artemisinin onto silica gel as supporting matrix, and vinyltriethoxysilane grafted onto its surface. For the formation of MIPs artemisinin was applied as template, acrylamide and methacrylic acid as the functional monomers, ethylene glycol dimethacrylate as the cross-linker and 2,2-azo-bis-isobutyronitrile as the initiator.

Radhapyari et al. [7] investigated a bioactive electrode prepared by dispersing graphene oxide in polyaniline composite, immobilized with horseradish peroxidase (HRP) electrochemically onto an indium tin oxide substrate, and used it for the determination of artesunate in human serum, plasma and urine. Phukon et al. [22] reported the investigation of a polyhydroxyalkanoate–gold nanoparticle composite with HRP enzyme adsorbed and immobilized on an indium–tin oxide glass plate (PHA/AuNPs/HRP/ITO) based biosensor for electrochemical determination of artemisinin in bulk and in spiked human serum.

He et al. [23] developed an easy-to-use lateral flow dipstick immunoassay for qualitative and semi-quantitative analysis of artesunate and dihydroartemisinin in anti-malarial drugs based on a monoclonal antibody (mAb) raised against artesunate. Ranjbar and coworkers [24] investigated different *Artemisia* species to analyze the artemisinin content, as well as the expression levels of genes responsible for the artemisinin biosynthetic pathway at different developmental stages.

For the quick and reliable quantification of special active substance derived from *Artemisia* herbs and its derivatives, new type of immunosensor based on OWLS detection was investigated. Since this label-free detection method has been successfully applied for the investigation of different immunosensors for the determination of a number of different compounds [25–29], our task was to apply this technique also for the analysis of the active compounds of *Artemisia* herbs and antimalarial drugs.

2. Materials and methods

2.1. Reagents, plant materials

Monoclonal antibodies against artemisinin, artemether and artesunate, antigens, standards and the conjugates for the sensitization of the sensors were prepared as reported earlier [17,18,23]. All other chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), unless otherwise stated.

Sweet wormwood (*A. annua*) plants were collected from East China (Anhui and Shandong provinces) and in Hungary (near Budapest), common wormwood (*A. vulgaris*), white wormwood (*A. alba*), absinthe wormwood (*A. absinthinum*), Japanese mugwort (*A. princeps*) and common ragweed (*Ambrosia artemisiifolia*) plants were collected in the Central Hungary region (near Üröm, Pest County). In addition, a sample of *A. vulgaris* was obtained commercially from Fitodry Ltd.

2.2. Sample preparation

The freshly picked plants, dissected to vegetal parts, were dried carefully using Ezidri Ultra 1000 food dehydrator (Hydraflow Industries Ltd., Wallaceville, New Zealand) for 24 h, and the different plant parts were ground separately. Three hundred mg of