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Challenging genosensors in food samples: The case of gluten determination in highly processed samples

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ABSTRACT

Electrochemical genosensors have undergone an enormous development in the last decades, but only very few have achieved a quantification of target content in highly processed food samples. The detection of allergens, and particularly gluten, is challenging because legislation establishes a threshold of 20 ppm for labeling as gluten-free but most genosensors expresses the results in DNA concentration or DNA copies. This paper describes the first attempt to correlate the genosensor response and the wheat content in real samples, even in the case of highly processed food samples. A sandwich-based format, comprising a capture probe immobilized onto the screen-printed gold electrode, and a signaling probe functionalized with fluorescein isothiocyanate (FITC), both hybridizing with the target was used. The hybridization event was electrochemically monitored by adding an anti-FITC peroxidase (antiFITC-HRP) and its substrate, tetramethylbenzidine. Binary model mixtures, as a reference material, and real samples have been analyzed. DNA from food was extracted and a fragment encoding the immunodominant peptide of α2-gliadin amplified by a tailored PCR. The sensor was able to selectively detect toxic cereals for celiac patients, such as different varieties of wheat, barley, rye and oats, from non-toxic plants. As low as 0.001% (10 mg/kg) of wheat flour in an inert matrix was reliably detected, which directly compete with the current method of choice for DNA detection, the real-time PCR. A good correlation with the official immunoassay was found in highly processed food samples.

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1. Introduction

Celiac disease is an autoimmune disorder of the small intestine that occurs in genetically predisposed people. The inflammatory response is triggered by the gluten fraction, present in wheat, a staple food which belongs to the Poaceae family [1], but also in closely phylogenetically related cereals such as rye and barley [2], which have a common ancestral origin in the grass family [3]. Oats, more distantly related to wheat, could also trigger the typical symptoms, although its harmful role is actually a term of debate and celiac patients are advised to avoid its consumption [4,5]. It is also possible to find gluten in the durum wheat, primarily used in the manufacture of pasta products, and in more ancient and less frequently consumed species, such as spelt, whose market is booming with the increasing demand of organic and natural products. Considering their harmful effect in celiac patients, the complete avoidance of gluten-containing cereals in their diet is desirable [6], but extremely difficult. For this reason, the term "gluten-free" is presently applicable to food products containing less than 20 mg/kg of gluten [7], as a tradeoff between the concentration reliably detected by the current analytical methodology and the safety of most celiac individuals.

Despite the legislation, no specific analytical methodology has been recommended for gluten monitoring yet. Most employed methods are based on enzyme linked immunosorbent assays directed to different gluten protein fractions [8,9]. Recently, aptamer based approaches [10] and electrochemical immunochips [11] have appeared with increased sensitivity.

Due to the high stability of DNA molecules compared to proteins, methods relying on DNA have also attracted great attention.

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Several articles have appeared in the last two decades to quantify gluten content in foodstuff based on the real-time polymerase chain reaction (PCR) [12–17]. Despite the sensitivity and fast-analysis of real-time PCR methods in gluten analysis, the need for expensive, complex and delicate optical instruments precludes its wide implementation in medium and small lab facilities as those available in food industry. Aiming at developing a portable, cheap and simple technology for DNA analysis, efforts have been done to replace the optical detection by non-optical techniques, which are more robust, less expensive and easier to miniaturize.

As alternative systems to detect DNA, electrochemical genosensors have arisen. They rely on a hybridization recognition reaction between two DNA complementary strands: the target and a recognition element, called probe. These devices use an electrode-based platform as transducer and, in order to convert this highly specific event into a measurable signal, a reporter molecule is usually incorporated, e.g. redox enzymes. After more than a decade of development, few electrochemical genosensors have been challenged to real food samples with quantitation purposes [18]. Analyzing DNA in food samples requires a mandatory pretreatment, which typically includes extraction of DNA, amplification, usually by PCR, and a subsequent post-PCR purification step [19]. In addition to this, the usual way of expressing the result is in DNA concentration units, which is meaningless for allergic consumers, and for verifying the compliance with the legislation.

In this paper, the challenge of facing an electrochemical genosensor to food samples is first addressed. The analytical performance under realistic conditions is evaluated in term of wheat percentage from which a correlation with legal threshold can be obtained to allow the verification of the labeling. Detection of other toxic cereals for the determination of total gluten content was also evaluated along with cross-reactivity against non-toxic rice and soy. Finally, processed food samples, with a wide range of expected gluten content were analyzed and validated against the Codex recommended method [20].

2. Experimental

2.1. Reagents

Dithiothreitol (DTT), 6-mercapto-1-hexanol (MCH), tetramethylbenzidine (TMB) in a ready-to-use reagent format (K-blue enhanced activity substrate, also containing H_2O_2) and saline sodium phosphate–EDTA (20 × SSPE pH 7.4) were purchased from Sigma-Aldrich (Spain). 1% Casein buffer in 1 × PBS was obtained from Fischer Scientific (Spain). Anti-fluorescein-HRP Fab fragments (antiFITC-HRP) were received from Roche Diagnostics GmbH (Mannheim, Germany). Ethanol was purchased from Panreac (Spain). NAP-10 columns of Sephadex G-25 were obtained from Life Technologies (Spain). Water was purified with a Milli-Q system (Millipore, Spain).

Oligonucleotide sequences used are shown in Table 1. Oligomers were obtained as lyophilized desalted salts from Sigma-Life Sciences. These sequences were selected to detect a sequence of 134-nt that encodes a highly toxic protein from wheat for celiac patients, the α2-gliadin, Gli [21], a multi-copy gene, which could provide a better performance in terms of sensitivity, compared to single-copy genes. Two probes, CP-Gli and SP-Gli, which hybridize with the entire target sequence were previously designed [22]. To ensure the quality of the extracted DNA for PCR amplification, the nuclear 18S rRNA gene was amplified as a universal control [23]. All stock solutions were prepared in MilliQ water and all reagents were used without any treatment except for CP-Gli that was unprotected as indicated elsewhere [22].

Table 1
Target, probes and primers sequences used.

Sequence name	Length	Oligonucleotide sequences 5′→ 3′
Signaling probe (SP-Gli)	63 nt	CTG CGG ATA TGG TAG TTG CGG CTG CGG ATA TGG TAG TTG CGG CTG CGG AAA TGG TTG CAG CTG-FITC
Capture probe (CP-Gli)	71 nt	HS-$(CH_2)_6$-TAC TGT GGT TGC GAT TGT GGA TAT GGT TGT TGT GGT CGA AAT GGT TGC GGC TGC GGA TAT GGT AGT TGC GG
Gli forward primer (Gli-F)	21 nt	CAG CTG CAA CCA TTT CCG CAG
Gli reverse primer (Gli-R)	23 nt	TAC TGT GGT TGC GAT TGT GGA TA

2.2. Instrumentation

Electrochemical measurements were carried out with screen-printed gold electrodes (SPEAu, DropSens-220BT, Spain), connected to a PGSTAT101 potentiostat with NOVA 1.9 software (EcoChemie, The Netherlands). The layout of the disposable planar screen-printed gold electrodes includes three electrodes in the same alumina sheet: a working gold electrode (Ø ~4 mm), an Ag pseudo-reference electrode and a gold counter electrode, all of them screen-printed on a ceramic substrate. The PCR reactions were performed in an Eppendorf Mastercycler thermocycler (Eppendorf, Hamburg, Germany).

2.3. Sample preparation

Wheat flour was mixed with rice flour to obtain different percentages of wheat from 50% down to 0.0001%. The most concentrated mixture (50%) was prepared by mixing 50 g of rice flour with 50 g of wheat flour. The rest of mixtures were prepared by successive dilution with rice flour to obtain a final amount of 100 g in separated recipients ensuring that no carry-over DNA was present from previous experiments by using a mixture of bleach and detergent and hot water, and thoroughly homogenized in a mixer.

Flours from different plants (bread wheat, durum wheat, spelt, barley, rye, oat, soybean and rice) were obtained at local markets. To minimize cross-contaminations among samples, the preparation of cereals, food samples and reference mixtures were carried out on different days and immediately stored at −20 °C until its use for DNA extraction.

2.4. DNA extraction

DNA was extracted from 200 mg of sample using the Nucleospin Food kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with minor alterations as described by Martín-Fernández et al. [17]. UV spectroscopy measurements (UV260 Shimadzu, Japan) were used to control the yield and purity of extracts (260/280 nm ratio around 1.8) and to estimate the DNA concentration extracted from which the working solution (50 ng/L) was prepared.

2.5. PCR amplification conditions

Unless otherwise indicated, the end-point PCR amplifications were carried out in 25 μL of total reaction volume containing 100 ng of DNA (2 L), 200 μM of each dNTP, 200 nM of each Gli primer (Gli-F/Gli-R), 1.5 mM of $MgCl_2$, 67 mM of Tris–HCl (pH 8.8), 16 mM of $(NH_4)_2SO_4$, 0.01% Tween 20, and 1.0 U SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany). Amplification conditions were: initial step at 95 °C for 10 min; 40 cycles at 95 °C

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