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An electronic tongue for gliadins semi-quantitative detection in foodstuffs

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ABSTRACT

An all-solid-state potentiometric electronic tongue with 36 polymeric membranes has been used for the first time to detect gliadins, which are primarily responsible for gluten intolerance in people suffering from celiac disease. A linear discriminant model, based on the signals of 11 polymeric membranes, selected from the 36 above using a stepwise procedure, was used to semi-quantitatively classify samples of a "Gluten-free" foodstuff (baby milked flour), previously contaminated with known amounts of gliadins (<10, 20–50 or >50 mg/kg), as "Gluten-free", "Low-Gluten content" or "Gluten-containing". For this food matrix, the device had sensitivity towards gliadins of 1–2 mg/kg and overall sensitivity and specificity of 77% and 78%, respectively. Moreover, the device never identified an ethanolic extract containing gliadins as "Gluten-free". Finally, the system also allowed distinguishing "Gluten-free" and "Gluten-containing" foodstuffs (15 foods, including breads, flours, baby milked flours, cookies and breakfast cereals) with an overall sensitivity and specificity greater than 83%, using the signals of only 4 selected polymeric membranes (selected using a stepwise procedure). Since only one "Gluten-containing" foodstuff was misclassified as "Gluten-free", the device could be used as a preliminary tool for quality control of foods for celiac patients.

1. Introduction

Celiac disease (CD), also known as gluten-sensitive enteropathy, is an autoimmune-mediated disorder, which is triggered in genetically susceptible individuals by the ingestion of gluten proteins of wheat, rye, barley and possibly oats. These proteins can be divided into glutenins and prolamins which are insoluble and soluble in aqueous alcohol solutions, respectively [1–3]. Population-based studies have shown that the prevalence of CD is in the range of 0.5–1.0% in Europe and US [4–8]. After consuming gluten, people who suffer from CD undergo a chronic inflammation of the small upper intestine mucosa, which can decrease the absorption of essential nutrients (e.g., iron, folic acid, calcium and fat soluble vitamins) and be a risk factor for other chronic diseases (e.g., type 1 diabetes, autoimmune thyroid disease, autoimmune liver disease, rheumatoid arthritis, Addison's disease, and Sjögren's syndrome, intestinal cancer, osteoporosis, female infertility, neurological and psychiatric disorders) [3,5,6,8–17]. The disturbances may revert, if a strict "Gluten-free" lifelong diet is established, the only treatment available nowadays [2,3,10,15,17]. The availability of "Gluten-free" or "Low-Gluten content" foodstuffs is therefore crucial for the quality of life of CD patients. However, commercial food products declared as "Gluten-free" can be often contaminated with gluten [18,19] and, therefore, dietary compliance is not always efficient. In order to prevent these cases, after the 1st of January of 2012, all EC state members must label foods as "Gluten-free" (<20 mg gluten/kg) or "Low-Gluten content" (20–100 mg gluten/kg) [20] (according to the European legislation that requires an obligatory labelling of ingredients that can cause adverse reactions [21]).

Therefore, to certify "Gluten-free" products, high detectability assays are required [2,3,22]. Gluten determination is usually made by quantification of gliadins, which are prolamins, being the content taken as 50% of gluten, by immunochemical assays, after extraction into ethanol/water solutions (from 60:40 to 70:30, v/v) [23–28]. Then, the gluten content has to be calculated by multiplying the gliadins content by a factor of 2, although this calculation

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is questionable and depends of several factors, namely the type of foodstuff under analysis [27].

In the literature, different analytical methods have been proposed to detect gliadins contents, such as: acid- and sodium dodecyl sulfate-polyacrylamide gel electrophoresis [19,29,30], capillary electrophoresis [23,31], flow cytometry [4], fluorescence correlation spectroscopy [3], fluorimmunoassays and fluorimetric methods [23], immunologically based sandwich or competitive enzyme-linked immunosorbent assay techniques [32–34], matrix-assisted laser desorption ionisation/time-of-flight mass tandem spectrometry [1,2,5,23], polymerase chain reaction systems [9,14,17,21], and reversed-phase and size-exclusion high-performance liquid chromatography [7,23,29–31,35]. Among them, immunochemical methods are the most used tools [7], ELISA tests with the monoclonal anticorp for ω-gliadin being the officially accepted method by the Association of Official Analytical Chemistry [36,37]. However, in general, these methodologies are time-consuming, require non-portable high-cost equipments that need very strict controlled operating conditions and high-qualified trained personal, and usually use large amounts of consumables.

Thus, there is a strong need for the development of low-cost, fast, sensitive and user-friendly integrated green analytical systems to detect gliadins in foodstuffs. CD diagnosis based in biosensors has been discussed in the literature, for instance, the use of an impedimetric immunosensor for the detection of autoantibodies directed against gliadins [15] and the implementation of an electrochemical immunosensor for the diagnosis of celiac disease, based on the detection of antibodies to tissue transglutaminase in human serum [8]. However, few works dealing with the development of gluten sensors for food analysis have been published [2,22]. Therefore, the development of an electronic tongue (E-tongue) for the detection of gliadins in foods represents a perspective worth pursuing, namely to be applied for discriminating "Gluten-free" from "Gluten-containing" foods.

The signal profiles recorded by E-tongue devices, composed by one or more arrays of sensors, together with chemometric techniques, are usually used to characterize samples qualitatively (by recognition and classification), but recently efforts to use the devices for semi-quantitative or even quantitative determination of liquid complex matrices have been developed [38–40]. The design of E-tongues may be carried out taking in account a wide variety of measuring techniques and chemical sensors [40]. The application of E-tongues (potentiometric, voltametric, impedimetric as well as approaches embracing mass and optical-sensors) in the food area has raised an increasing interest, mainly due to the simplicity and low cost of the equipment and easiness of utilization, low calibration requirements, satisfactory accuracy and easy adaptability to different working conditions, when compared with other analytical methodologies [41,42]. This kind of devices has been widely used for analysis of several kinds of foods, namely, mineral water [43], tea [44], milk [45,46], soft drinks and juices [38,39,47–49], wine [50,51], beer [52], honey [53] and plants [54]. A recent review of the advances in the field of electronic tongues employing electroanalytical sensors as well as biosensors, mainly enzyme-based, summarizes the most important results and real applications [55].

In this work, an all-solid-state potentiometric multisensors device with polymeric membranes based on lipid derivative additives was prepared and applied to discriminate between "Gluten-free" and "Gluten-containing" foods. The sensor features of these polymeric membranes are different from those of the traditional high selective chemical sensors, the goal being to obtain global information about the solution through non-specific chemical sensors with low selectivity and high cross-sensitivity to different species [39].

The main aim of this work was to evaluate the differentiation between "Gluten-free" and "Gluten-containing" foods with an E-tongue constituted by 36 lipo/polymeric membranes and a linear discriminant model, by relating the signals profile recorded with the gliadins content of ethanolic extracts. First, to evaluate the E-tongue performance, the expected food matrix influence on signal patterns recorded by the device was evaluated by assessing its ability to distinguish the ethanolic extracts of the different foodstuffs studied, regardless their gliadins content. Subsequently, the capability of the E-tongue to semi-quantitatively detect gliadins levels (<10 mg/kg up to 200 mg/kg) in ethanolic extracts from different samples of a "Gluten-free" foodstuff contaminated, or not, with known gliadins amounts, was verified. Finally, the potential of the device to discriminate "Gluten-free" from "Gluten-containing" commercial foodstuff's samples (the label information was checked by HPLC-DAD analysis), based on the signal patterns recorded after analysing their ethanolic extracts, was assessed.

2. Material and methods

2.1. Sampling

Fifteen commercially available foodstuffs, 8 labelled as "Gluten-containing" and 7 as "Gluten-free" (flours, baby milked flours, breads, cookies and breakfast cereals), were purchased at local supermarkets in Bragança, Portugal. According to the label information, "Gluten-free" foods were obtained mainly from rice and/or maize (baby milked-flour); rice, maize, soya and/or lupine (cookies); buckwheat flour and maize (flour); maize (breakfast cereals) and buckwheat flour, rice and maize (bread). The "gluten-containing" foods were mainly obtained from wheat, although some of them (baby milked-flour and breakfast cereals, respectively) also contained maize, barley, oats, maize, rice and/or rye. All the foodstuffs, except flours and baby milked flours, were milled and converted to powder before use.

2.2. Reagents, gliadins standard and sample preparation

All chemicals and reagents were of analytical grade and used as supplied. A gliadins standard from wheat supplied by Sigma (ref. G3375) was used as a basis for standardizing the analysis and detection of gliadins. Acetonitrile (Labscan) with a minimum purity of 99.8% was supplied by Merck. Trifluoroacetic acid with a minimum purity of 99% was provided by Acros Organics. Absolute ethanol was purchased from Panreac. All aqueous solutions were prepared with deionized water obtained with a TGI Pure Water System. All the polymeric membrane components were also of analytical grade, obtained from Fluka and used as purchased.

Gliadins stock solutions were freshly prepared by dissolving pre-established gliadins minced masses in 70% (v/v) ethanol. The masses were weighted using an analytical KERN balance (model ASS 220-4, ±0.1 mg). The solutions were then shaken during 15 min in a vortex (model VM2 Cat 230 V) and finally centrifuged during 10 min at 5000 rpm, using a Centurion K_2R Series refrigerated centrifuge, for removing undissolved material. The protein concentration of the supernatant of each gliadins stock solution was determined using the "Coomassie Plus – The Better Bradford™ Assay Kit" from Pierce, according to the manufacturer's instructions. A BioTek plate reader (model ELx800) was employed and the absorbance of each solution was recorded at 595 nm. The concentrations of the standard solutions determined by the Bradford method [56] were checked against the values expected assuming total dissolution of the added gliadins masses.

Two different gliadins extraction procedures were applied to the foodstuffs, both based on Bietz et al. [57]. In the first, four samples (100 g each) of the same "Gluten-free" foodstuff (according to the label and confirmed by HPLC analysis) were contaminated

with pre-established amounts (from 4 up to 20 mg) of gliadins standard and then extracted. In the second, gliadins were extracted from three samples of each one of the 15 foodstuffs (10 "Gluten-containing" and 5 "Gluten-free", according to HPLC analysis).

2.2.1. Procedure 1

Known amounts of gliadins standard powder were added to four samples of a baby milked flour food to obtain samples with pre-established amounts of gliadins: 20 mg/kg (4 mg of gliadins powder into 200 g of "Gluten-free" food), 50 mg/kg (5 mg/100 g), 100 mg/kg (10 mg/100 g) and 200 mg/kg (20 mg/100 g). Each contaminated sample was replicated five times. Before use, each sample was shaken during 10 min in a vortex. As controls, 10 samples of the same "Gluten-free" food without any gliadins addition were also used. From each sample an amount of *ca.* 4 g was removed and extracted with 40 mL of 70% (v/v) aqueous ethanol, being shaken for 15 min in a vortex (model VM2 Cat 230 V). The suspensions obtained were kept overnight at 4 °C and analysed in the following day with the E-tongue, after shaking in the vortex to reinforce the dissolution process.

2.2.2. Procedure 2

From each foodstuff studied, an amount of approximately 4 g (flours) or 12 g (other foods) of powder was extracted with 60 mL of 70% (v/v) aqueous ethanol with magnetic stirring (VELP Scientific magnetic stirrer) for 30 min, at room temperature. The suspensions were centrifuged (Centurion K2R Series refrigerated centrifuge) for 10 min, at 5000 rpm and room temperature. The supernatants were stored at 4 °C until use. All of them were analysed by HPLC and by the E-tongue. The gliadins concentrations of each foodstuff were obtained by HPLC measurements of the ethanolic extracts, taking into account the dilution factor (15× and 5× for flours and the other foods, respectively).

2.3. Chromatographic conditions and HPLC-DAD analysis

A Varian HPLC system equipped with a Prostar 220 pump, a 7725i Rheodyne manual injector with a 10 μL loop and a ProStar 330 Photodiode Array detector (DAD) was used for LC separation. A Star Chromatography Workstation software (version 4.5) was used for data analysis. The chromatographic separation was achieved using a PLRP-S column (polystyrene divinylbenzene stationary phase, particle size 8 μm, pore 300 Å and 150 × 4.6 mm id) fitted with a PLRP-S security guard cartridge (5 mm × 3 mm id). The column was placed inside an oven (Jones, Model 7981) and kept at 40 ± 0.1 °C. The mobile phase consisted of eluent A, acetonitrile/TFA/water (1:0.01:99, v/v), and eluent B, acetonitrile/TFA/water (99:0.01:1, v/v). Proteins were eluted with a flow of 0.6 ml/min, at 40 ± 0.1 °C, using a linear gradient obtained by increasing the proportion of solvent B, from 20% to 65%, over 30 min. The detection was made simultaneously at four wavelengths (210, 220, 250 and 280 nm). For all HPLC analysis, the UV spectra were recorded between 190 and 400 nm. The UV spectrum of the chromatographic peaks obtained for each food sample was compared with those recorded for the standard gliadins. The peaks with different UV profiles were not included in the quantification procedure since they might correspond to other proteins. The solvents were filtered through 0.20 μm nylon filters (47 mm of diameter, from Millipore) and degassed for at least 30 min prior to use, in an ultrasound bath from Elma (model Transsonic 460/H). The ethanolic extracts of the gliadins standard solutions and of the samples were also filtered through 0.20 μm nylon filters (Puradisc 25 NYL with a diameter of 25 mm, from Whatman), being each extract analysed in triplicate. The method for the determination and quantification of the gliadins was validated by testing the linearity and the precision. The repeatability and intermediate precision were evaluated using relative square deviations, RSD. For repeatability evaluation, a gliadins standard solution (227 mg/dm^3) was analyzed five times in the same day and the intermediate precision was assessed by analysing the same solution seven times in each of three consecutive days. The external standard method was used to calibrate the chromatographic system for the gliadins quantification. For this purpose six standard solutions of gliadins with concentrations ranging from 50 to 1309 mg/dm^3 (confirmed using the Coomassie Plus – The Better Bradford™ Assay Kit) were used. The detection and quantification limits were determined from the parameters of the calibration curve, being defined as 3.3 and 10 times the value of the intercept error divided by the slope, respectively [58,59]. The detection limit was also evaluated by calculating the RSD of the gliadins peaks total area obtained after injecting 8 times an ethanolic standard solution with a concentration of 10 mg/dm^3 of gliadins.

2.4. Potentiometric E-tongue device

The E-tongue used in this work was described in previous works [38,39,45]. The device consisted of 36 polymeric membranes applied to two-sensor arrays, which are different from those usually used in ion selective electrodes. The membranes were prepared using organic compounds with long carbon chain with different functional groups (lipid additive compounds not selective to an ionic compound) being non-specific chemical sensors with low selectivity and cross-sensitivity to the different species in the samples [60] (both inorganic and organic, ionic and non-ionic). Therefore, each membrane was prepared with approximately 31.9–32.3% of polyvinyl chloride as polymeric matrix, 64.7–65.2% of one plasticizer compound (A: Bis(2-ethylhexyl)phthalate; B: Bis(1-butylpentyl) adipate; C: Tris(2-ethylhexyl)phosphate; D: Dibutyl sebacate; E: 2-Nitrophenyl-octylether; or, F: Dioctyl phenylphosphonate) and 2.8–3.2% of one sensor membrane additive (I: Octadecylamine; II: Bis(2-ethylhexyl)phosphate; III: Oleyl alcohol; IV: Methyltrioctylammonium chloride; V: Tridodecylmethylammonium chloride; or, VI: Oleic acid).

The multisensor system, together with a double junction Ag/AgCl reference electrode, was connected to a multiplexer Agilent Data Acquisition/Switch Unit model 34970A. An Agilent BenchLink Data Logger software installed in a PC computer was used to acquire the sensor signals.

Measurements were performed in a double wall glass cell thermostatized at 25 °C, being the signals pattern recorded after a 7 min analysis period.

2.5. Statistical analysis

Linear discriminant analysis (LDA) was used as a supervised learning technique, for treating the signals recorded by the E-tongue device. This technique was applied to: (i) differentiate between the foodstuffs analysed based on their ethanolic matrices; (ii) classify the ethanolic extracts of different samples from a "Gluten-free" foodstuff, contaminated with known gliadins amounts, according to their gliadins contents, into "Gluten-free", "Low-Gluten content" or "Gluten-containing" foods; and (iii) to distinguish "Gluten-free" from "Gluten-containing" samples of the different foodstuffs under study, regardless the effect of the food matrix. Prior probabilities were computed based on each group size. A stepwise technique, using the Wilk's lambda method with the usual probabilities of *F* for a variable to be included (0.05) or removed (0.10) from the model, was applied for variable selection. A variable is entered into the model if the significance level of its *F* value is less than the entry value and is removed if the significance level is greater than the removal value. This procedure uses a combination of forward selection and backward elimination pro-

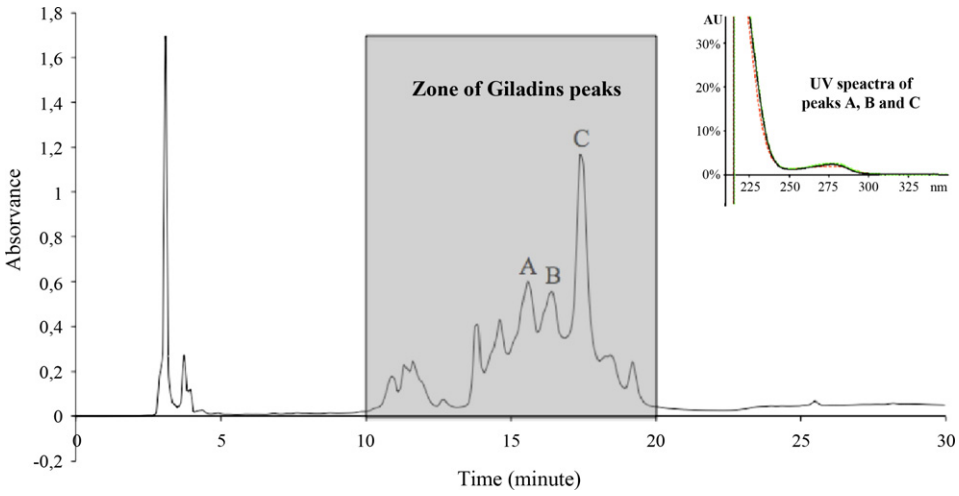

Fig. 1. Chromatographic profiles of gliadins peaks of ethanolic standard solution (584 mg/dm^3) recorded at 210 nm, using HPLC-DAD (10 μl injection).

cedures, where before selecting a new variable to be included, it is verified whether all of the variables previously selected remain significant [61]. So, at each step, the variable that minimizes the overall Wilks' lambda is entered. With this approach it was possible to identify the significant variables (sensor signals) among the sensor signal profiles recorded by the E-tongue device for each sample. To verify which canonical discriminant functions were significant, the Wilks' Lambda test was applied. To avoid overoptimistic data modulation, a leaving-one-out cross-validation procedure was carried out to assess the model performance. The LDA statistical analysis was performed at a 5% significance level using the SPSS software, version 17.0 (SPSS Inc.) and the JMP software, version 8.0.2 (SAS Institute, UK).

3. Results and discussion

3.1. Separation and quantification of gliadins by HPLC-DAD

The HPLC conditions were optimised for wavelength, mobile phase composition, gradient, operating temperature and flow-rate. The best results concerning the identification and quantification of the major eluted peaks of the gliadins standard solutions were achieved at 210 nm. A typical chromatographic profile recorded for an ethanolic gliadins standard solution, in Fig. 1, shows that gliadins peaks were eluted in the retention time range of 10–20 min. The linearity of the method, for calculation of the whole gliadins content in ethanolic extracts, was checked through a calibration curve obtained by linear regression (R = 0.9993), considering the total peaks areas in arbitrary units (Fig. 1), versus the concentration of gliadins (mg/kg).

The limits of detection (LOD) and quantification (LOQ), calculated from the calibration parameters [58,59], were 46.5 and 140.9 mg/kg gliadins, respectively. As the chromatogram visualization showed that the LOD should be smaller, its value was further assessed by RSD-based verification, by injecting 8 times a standard solution of 10 mg/dm^3. In these assays, the RSD for total area of the peaks detected was equal to 6.6%. This result shows that the LOD value could be set equal to 10 mg/kg or even lower (LOD can be defined as the minimum concentration below which the RSD exceeds 17%, [59]). This fact was further confirmed since the chromatograms recorded for the standard solution of 10 mg/dm^3, showed at least one peak in the typical retention time range of gliadins that had the expected typical gliadins UV spectrum profile (from 190 to 340 nm, Fig. 1).

The precision of the HPLC method was checked by evaluating the repeatability and the intermediate precision. The RSD for the within-day and between-day variations were equal to 2.5% and 3.5%, respectively. Therefore, the method showed satisfactory precision, since the RSD values were lower than 5% [58].

The calibration curve was used to quantify the gliadins contents in the foods samples (flours, baby milked flours, bread, cookies and breakfast cereals), to confirm if the "Gluten-free" labelled foods were not contaminated with gluten and to infer about the levels of gliadins in the "Gluten-containing" labelled foods. In Table 1, the average gliadins concentrations for the ethanolic extracts of each food analysed are presented, as well as the gliadins concentrations in the food samples, which were calculated considering the dilution factor for each type of food (15× and 4× for flours and the other foods, respectively). The results showed that two "Gluten-free" labelled foods were contaminated with gliadins and therefore, in the present study, they were considered as "Gluten-containing" products. Furthermore, it could also be inferred that the gliadins content of the three different ethanolic extracts obtained for each foodstuff are in good agreement (RSD values lower than 13%), showing that the extraction procedure adopted has satisfactory repeatability. Finally, the gliadins concentrations of the "gluten-containing" flours studied (around 11 g/kg, for not dried flour) are in agreement with those reported by Ciclitira et al. [62] for flours, which vary from 1.2% to 3.3% of dry matter. However, these contents are quite lower than those reported by DuPont et al. [30], which were 4%.

3.2. Electronic tongue analysis

In previous works [38,39,45], a similar multisensor system, made up of cross-sensitivity polymeric membranes, has been applied as a taste sensor for qualitative, semi-quantitative and quantitative analysis in food matrices. In this work, the E-tongue was used for analysing ethanolic extracts (70:30 ethanol/water, v/v) of different foods and to infer if the system could detect the presence of gliadins, since it was expected that E-tongue signals would be influenced by the food matrices and consequently by the differences of the ethanolic extracts matrices. The sensors signal stability towards solutions containing 70% of ethanol was evaluated. The average sensor signal drift value obtained during 30 min after 10 min of stabilization was lower than 1.5 mV. This result showed that the polymeric membranes were suitable for analysis of these ethanolic solutions.

Table 1
Average gliadins concentrations of the ethanolic extracts of each food analysed by HPLC-DAD at 210 nm, and the respective foodstuff concentration.

Product label	Foodstuff	Ethanolic extract		Food sample
		Gliadins concentration (mg/dm^3)[a]	RSD (%)	Gliadins concentration (g/kg)
"Gluten-containing"	Flour (Brand 1)[b]	760	5	11.3
	Flour (Brand 2)[b]	706	7	10.6
	Bread (Brand 1)[b]	624	2	3.11
	Baby milked flour (Brand 1)[b]	220	1	1,09
	Baby milked flour (Brand 2)[b]	601	13	2.98
	Cookies (Brand 1)[b]	d	–	–
	Cookies (Brand 2)[b]	d	–	–
	Breakfast cereals (Brand 1)[b]	d	–	–
"Gluten-free"	Flour (Brand 3)[b]	d	–	–
	Bread (Brand 2)[c]	nd	–	–
	Baby milked flour (Brand 3)[b]	187	11	0.930
	Baby milked flour (Brand 4)[c]	nd	–	–
	Cookies (Brand 3)[c]	nd	–	–
	Cookies (Brand 4)[c]	nd	–	–
	Breakfast cereals (Brand 2)[c]	nd	–	–

[a] d, gliadins detected (>10 and <150 mg/dm^3); nd, gliadins not detected (<10 mg/dm^3).
[b] Samples designated as "Gluten-containing" foods based on the HPLC results.
[c] Samples designated as "Gluten-free" foods based on the HPLC results.

E-tongue devices have been used successfully to classify food matrices based on their physico-chemical and sensorial characteristics [39,43,45–53]. In this study, the E-tongue signal pattern, recorded for the 45 ethanolic extracts, together with a LDA model, was able to successfully differentiate 5 different kinds of foodstuffs regardless their gluten level: breads (2 brands), breakfast cereals (2 brands), cookies (4 brands), flours (3 brands) and baby milked flours (4 brands). This discrimination capability of the device can be ascribed to the influence of the food matrix in the device performance, as can be visualised from Fig. 2. This figure shows a 3D graph considering the first 3 of the 4 significant discrimination functions (100% of the total variance explained, $P < 0.001$) established, based on the signals of 11 polymeric membranes (plasticizer-additive compound combination [39]: A-II, A-III, B-I, B-II, B-III, B-VI, D-I, D-II, E-II, E-III and E-IV), selected from the 36 polymeric membranes that were used in the E-tongue, by a stepwise method.

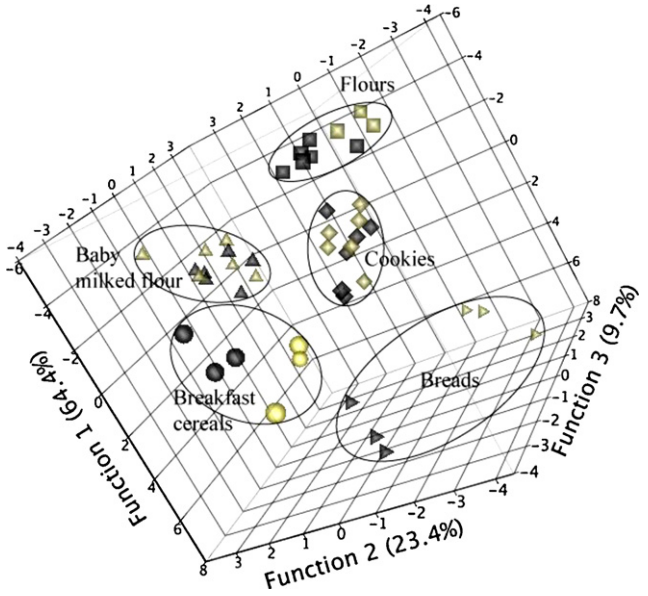

Fig. 2. Scores for the three main discriminate functions (percentage of variance explained in parentheses) based on the E-tongue signals of the ethanolic extracts of the 15 analysed foodstuffs: breads (2 brands, 6 samples), breakfast cereals (2 brands, 6 samples), cookies (4 brands, 12 samples), flours (3 brands, 9 samples) and baby milked flours (4 brands, 12 samples). (Filled dark markers: "Gluten-containing" samples; Filled light marks: "Gluten-free" samples).

Globally, the LDA model allowed 89% overall correct classifications according to the type of food, for the "leaving one-out" cross-validation procedure, regardless their gluten content. All bread samples were correctly classified and for all the other foodstuffs, only one sample was misclassified. These results strongly suggest the existence of a food matrix effect and therefore it is expected that the E-tongue signals are influenced by the differences between the ethanolic extract matrices. In accordance, the work to demonstrate the ability of the device for gliadins content evaluation was developed in two steps. First, the ability of the system to detect and semi-quantitatively differentiate gliadins levels in ethanolic extracts of a selected "Gluten-free" product, previously contaminated with known gliadins amounts, was evaluated. Using the same foodstuff, the matrix effect was avoided, and therefore differences between the E-tongue signal patterns must be due to the different gliadins contents. Secondly, the potential of the potentiometric system array to differentiate the 15 foodstuffs into "Gluten-free" and "Gluten-containing" foods (according to the HPLC-DAD results), independently of the food matrix, was evaluated using the ethanolic extracts. From the results shown in Fig. 2, this potential was expected since the system was able to distinguish from "Gluten-free" (open markers) and "Gluten-containing" (filled markers) of breads or breakfast cereals samples, even when influenced by the food matrix.

3.2.1. *E-tongue semi-quantitative gliadins evaluation in ethanolic extracts from samples of a "Gluten-free" foodstuff*

The E-tongue signal patterns of 30 ethanolic extracts, of different samples of the same "Gluten-free" foodstuff (baby milked flour) previously contaminated with different known gliadins amounts, were randomly recorded: 10 "Gluten-free" extracts (<10 mg gliadins/kg), 10 "Low-Gluten content" extracts (20–50 mg gliadins/kg) and 10 "Gluten-containing" extracts (>50 mg gliadins/kg). Exposing the device to the ethanolic extracts resulted in changes in the signal profiles recorded by the E-tongue showing that the polymeric membranes used were affected by the different gliadins contents. The signals recorded were analysed using a LDA model. Two significant discriminant functions accounting 100% of the total variance, were established ($P < 0.001$), based on the signals of also 11 polymeric membranes (plasticizer-additive compound combination [39]: A-III, A-V, B-II, B-III, B-V, B-VI, C-II, C-IV, D-III, E-I, F-II) selected by a stepwise method. In Fig. 3, the scores for the two functions are plotted (explaining 76.3% and 23.7% of the total variance, respectively). The results

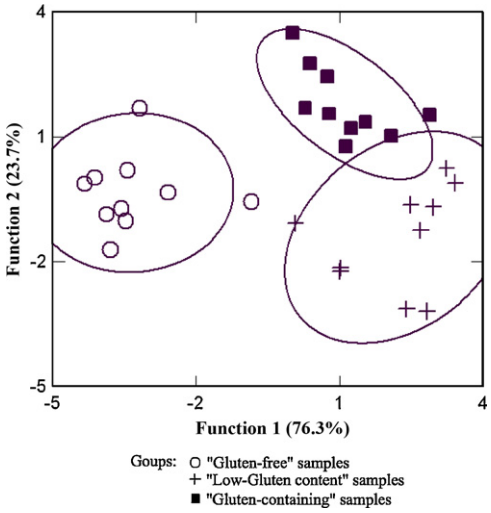

Fig. 3. Ethanolic extracts LDA classification into three groups obtained from samples of a "Gluten-free" baby milked flour: "Gluten-free" (<10 mg/kg gliadins), "Low-Gluten content" (20–50 mg/kg gliadins) and "Gluten-containing" (>50 mg/kg gliadins). Percentages of variance explained for each discriminant function are given in parentheses.

showed that this methodology is quite satisfactory, specially for differentiating ethanolic extracts of the same foodstuff with gliadins levels lower than 10 mg/kg ("Gluten-free" samples) from those with gliadins levels greater than 20 mg/kg ("Gluten-containing samples"). In Table 2, the classification results obtained using the "leaving one-out" cross-validation procedure are presented. The model performance allowed 77% overall correct classifications for the "leaving one-out" cross-validation cases. Also, for each group under study, the minimum sensitivity and the specificity were equal to 70% and 64%, respectively.

Although the model misclassified some samples for the cross-validation procedure (Table 2), it never identified a "Gluten-containing" ethanolic extract as "Gluten-free", which suggests that the E-tongue may be useful for quality control of foods for celiac patients. Since the gliadins concentrations in the ethanolic extracts were approximately 10 times lower than those of the food samples, it can be inferred that the E-tongue device has a sensitivity of 1–2 mg/kg, which corresponds to 2–4 mg gluten/kg. This result is very satisfactory since an analytical method with a sensitivity of 10 mg/kg per species is usually considered suitable for gluten detection [21]. Nevertheless, it should be referred that this result was obtained for a single food matrix, namely baby milked flour, and therefore, this conclusion could be overoptimistic, especially if different food matrices were considered simultaneously.

3.2.2. E-tongue discrimination between "Gluten-free" and "Gluten-containing" foods

The E-tongue device used allowed to record different signal patterns for the ethanolic extracts of the 45 foodstuffs samples (3 samples from each one of the 15 different foods). Although the signal patterns were affected by each food matrix, as already discussed, the ability of the E-tongue to discriminate "Gluten-free" from "Gluten-containing" real food samples was evaluated. According to the HPLC-DAD results (Table 1) and the EC rules for food labelling [20], the 15 foodstuffs were divided into "Gluten-free" (5 foods with gliadins contents lower than 10 mg/kg according to the HPLC results of Table 1: bread-brand 2; baby milked flour-brand 4; cookies-brands 3 and 4; and breakfast cereals-brand 2) and "Gluten-containing" foods (10 foods with gliadins contents greater than 50 mg/kg according to the HPLC chromatographic profiles recorded (data not shown) and the HPLC results of Table 1: flours-brands 1 to 3; bread-brand 1; baby milked flours-brands 1 to 3; cookies-brands 1 and 2; and, breakfast cereals-brand 1). The LDA used only one discriminant function ($P < 0.001$), which accounted for 100% of the total variance, based on the signals of only 4 polymeric membranes (plasticizer-additive compound combination [39]: B-V, C-I, C-V and D-I) selected from the 36 polymeric membranes that were used in the E-tongue, by a stepwise method. The model allowed to classify correctly 84% of the samples, for the "leaving one-out" cross-validation procedure, which is very satisfactory taking in account the higher complexity due to the food matrix effect. In addition, for each group under study, the minimum sensitivity and the specificity were of 73% and 79%, respectively, as can be seen from Table 3.

Furthermore, the cross-validation procedure leads to the misclassification of only one of the 10 "Gluten-containing" foodstuffs (3 samples of bread of brand 1) as "Gluten-free". This misclassification could be tentatively due to an additional difficulty in the gliadins extraction from bread, which is a more processed food matrix. Therefore, these results suggest that the E-tongue can be

Table 2
Contingency matrix obtained using LDA of the signals recorded by the E-tongue of the ethanolic extracts of different samples of the same "Gluten-free" foodstuff (baby milked flour, brand 4), with or without different added levels of gliadins standard (results from cross-validation procedure).

Actual group[a]	Predicted group			Total	Sensitivity (%)
	"Gluten-free"	"Low-Gluten content"	"Gluten-containing"		
"Gluten-free"	9	1	0	10	90
"Low-Gluten content"	0	7	3	10	70
"Gluten-containing"	0	3	7	10	70
Total	9	11	10	30	77
Specificity (%)	100	64	70	78	

[a] "Gluten-free" ethanolic extract: <10 ppm gliadins; "Low-Gluten content" ethanolic extract: 20–50 ppm gliadins; "Gluten-containing" ethanolic extract: >50 ppm gliadins.

Table 3
Contingency matrix obtained using the LDA of the signals recorded by the E-tongue for the ethanolic extracts of 45 samples of 15 foodstuffs, 5 "Gluten-free" and 10 "Gluten-containing", confirmed by HPLC-DAD (results from cross-validation procedure).

Actual group[a]	Predicted group		Total	Sensitivity (%)
	"Gluten-free"	"Gluten-containing"		
"Gluten-free" food samples	11	4	15	73
"Gluten-containing" food samples	3	27	30	90
Total	14	31	45	84
Specificity (%)	79	87	83	

[a] "Gluten-free" food samples: <10 ppm gliadins; "Gluten-containing" food samples: >50 ppm gliadins.

used as a preliminary tool for quality control of foods for celiac patients. Moreover, if this analysis was made for each kind of foodstuff, keeping a similar extract matrix, better results would be expected.

4. Conclusions

The detection of gliadins levels above 10 mg/kg in "Gluten-free" or above 50 mg/kg in "Low-Gluten content" foodstuffs is of main importance due to legal and healthy issues, being the last ones of major concern for CD patients. In this work, the feasibility of a simple, fast and low-cost solid-state potentiometric E-tongue for detecting gliadins in food ethanolic extracts, as well as to discriminate between "Gluten-free" and "Gluten-containing" foodstuffs samples, was demonstrated for the first time. The device was used directly in the centrifuged food ethanolic extracts and did not require any further pre-treatment step or the use of any other consumable (e.g., filters, columns, solvents or gases) and therefore constitutes a good example of practice of the green analytical chemistry of the future. The results obtained showed that the device: (i) had sensitivity around 1–2 mg/kg of gliadins for measurements on ethanolic extracts (value obtained for baby milked flour samples); (ii) could classify semi-quantitatively ethanolic extracts of foodstuffs with different gliadins contents (<10 mg/kg, 20–50 mg/kg and >50 mg/kg) with overall sensitivities and specificities greater than 77%, and it never classified an extract with more than 10 mg/kg of gliadins as an extract with less than 10 mg/kg of gliadins; and, finally, (iii) could distinguish with acceptable sensitivities and specificities (greater than 83%) "Gluten-free" from "Gluten-containing" food samples, regardless the food matrix effect, only one of the 10 "Gluten-containing" foods being misclassified. The results also showed that for both qualitative and semi-quantitative discriminations, the signals of the polymeric membranes made of plasticizers bis(1-butylpentyl) adipate or tris(2-ethylhexyl)phosphate (B or C) with amines and ammonium salts as addictive sensor substances (I, IV or V) were the main independent variables. This last finding was expected since it was reported that ammonium salts promoted protein binding [63,64].

Finally, the work carried out showed that the E-tongue could be used in practice as a fast and economic preliminary tool to evaluate, in a real time basis, the possible gluten contaminations of "Gluten-free" foodstuffs. Nevertheless, to use the E-tongue as a routine methodology for this purpose it is needed to improve the multisensor system by including some further non-specific sensors to proteins.

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