Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Analytica Chimica Acta 1140 (2020) 190–198

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Interconnectable solid-liquid protein extraction unit and chip-based dilution for multiplexed consumer immunodiagnostics

Georgina M.S. Ross [a], Daniel Filippini [b], Michel W.F. Nielen [a, c], Gert IJ. Salentijn [a, c, *]

[a] Wageningen Food Safety Research (WFSR), Wageningen University & Research, P.O. Box 230, 6700, AE, Wageningen, the Netherlands
[b] Optical Devices Laboratory, Division of Sensor and Actuator Systems, IFM – Linköping University, S58183, Linköping, Sweden
[c] Laboratory of Organic Chemistry, Wageningen University, Helix Building 124, Stippeneng 4, 6708 WE, Wageningen, the Netherlands

HIGHLIGHTS

- **1 min allergen protein extraction via disposable homogenizer and 3D-printed sieves.**
- **3D-printed unibody lab-on-a-chip (ULOC) for on-chip bioreagent storage and dilution.**
- **Pipette-free sample dilution and transport to multiplex immunosensor.**
- **Realtime smartphone analysis using 3D-printed device holder as a lightbox.**
- **Interconnectable and miniaturized biosensing system from sample to smartphone.**

ARTICLE INFO

Article history:
Received 13 July 2020
Received in revised form
25 September 2020
Accepted 9 October 2020
Available online 16 October 2020

Keywords:
3D-printing
Sample preparation
Smartphone-imaging
Unibody-lab-on-chip
Lateral flow immunoassay
Food allergen testing

ABSTRACT

While consumer-focused food analysis is upcoming, the need for multiple sample preparation and handling steps is limiting. On-site and consumer-friendly analysis paradoxically still requires laboratory-based and skill-intensive sample preparation methods. Here, we present a compact, inexpensive, and novel prototype immunosensor combining sample preparation and on-chip reagent storage for multiplex allergen lateral flow immunosensing. Our comprehensive approach paves the way for personalized consumer diagnostics. The prototype allows for handheld solid-liquid extraction, pipette-free on-chip dilution, and adjustment of sample concentrations into the appropriate assay dynamic working range. The disposable and interconnectable homogenizer unit allows for the extraction and 3D-sieve based filtration of allergenic proteins from solid bakery products in 1 min. The homogenizer interconnects with a 3D-printed unibody lab-on-a-chip (ULOC) microdevice, which is used to deliver precise volumes of sample extract to a reagent reservoir. The reagent reservoir is implemented for on-chip storage of carbon nanoparticle labeled antibodies and running buffer for dilution. The handheld prototype allows for total homogenization of solid samples, solid-liquid protein extraction, 3D-printed sieve based filtration, ULOC-enabled dilution, mixing, transport, and smartphone-based detection of hazelnut and peanut allergens in solid bakery products with limited operational complexity. The multiplex lateral flow immunoassay (LFIA) detects allergens as low as 0.1 ppm in real bakery products, and the system is already consumer-operable, demonstrating its potential for future citizen science approaches. The designed system is suitable for a wide range of analytical applications outside of food safety, provided an LFIA is available.

1. Introduction

On-site and personalized food safety tests are growing in popularity, with developments in rapid, affordable, sensitive, and disposable handheld assays driving the move from the laboratory to a consumer-based approach [1,2]. Consumer detection of food allergens is particularly relevant [3,4], and more so now than ever, with the Food and Drug Administration (FDA) announcing temporary changes to food labeling and allowing of ingredient alterations to prevent any disruption to the global food supply chain during the SARS-CoV-2 pandemic [5]. Amendments that overlook hidden or novel allergens put the allergic individual at risk,

* Corresponding author. Wageningen Food Safety Research (WFSR), Wageningen University & Research, P.O. Box 230, 6700, AE, Wageningen, the Netherlands
E-mail addresses: gert.salentijn@wur.nl, gert.salentijn@wur.nl (G.IJ. Salentijn).

https://doi.org/10.1016/j.aca.2020.10.018
0003-2670/

exemplifying the necessity for personalized, disposable, and simplified analysis of allergens, from sample preparation to detection. To date, the lateral flow immunoassay (LFIA) is the most successful application of consumer diagnostics [6]. Combining LFIAs with smartphones as optical detectors allow for 'on-the-go' decentralized screening [7] and smartphones can even provide semi-quantitative results by calibrating test and control line intensity values toward a particular antigen concentration [8].

Despite these advantages, LFIAs also have some disadvantages, including a limited dynamic range, they work only with liquid samples and predominately target only a single analyte. Within a sandwich LFIAs dynamic working range, the test line intensity increases alongside increasing analyte concentration. However, at high analyte concentrations, the signal intensity can paradoxically decrease as the excess of unlabeled analyte saturates the capture and detector antibodies (mAbs) binding sites [9]. The reduction in test line intensity can mimic the signal at a much lower analyte concentration. Dilution to within an assays appropriate concentration range is required to avoid false-negative results. False negatives are particularly problematic for consumers.

Moreover, when analyzing a complex solid matrix such as food, sample preparation, including homogenization of the solid food and extraction of the relevant proteins, as well as reagent storage, are pivotal bottlenecks. Even integrated systems often require pre-treatment [10] or heat-assisted actuation to extract proteins into a testable liquid [3]. Finally, excluding a few multiplex LFIAs [11–13], allergen LFIAs are restricted to singleplex detection, which is limiting for individuals with co-existing allergies. Sample preparation is a major issue; indubitably, consumers do not have the laboratory skills required for extracting, pipetting, and diluting samples, and fully integrated analytical systems have so far mainly been developed for DNA-based analysis [14–16]. Systems with integrated solid-phase extraction for aqueous samples are reported [10,17], but the extraction of solid samples is more complex and still requires offline pre-treatment.

In a parallel advancement, the emergence of 3D-printing has revolutionized the rapid prototyping of multifunctional lab-on-a-chip [18] and disposable [19] devices for analytical chemistry. Modification of Computer-Aided Designs (CADs) takes little cost and time, and prototypes can be refined iteratively multiple times in a single day, outside of a cleanroom environment. A unibody lab-on-a-chip (ULOC) [18,20] is a monolithic device with all the analytical functionalities in-built on one side, takes less than an hour to manufacture, and is printed in a single step [21]. The ULOC's unibody connectors, ending in unidirectional valves, can be connected to silicon tubing as manual finger pumps [22]; or to detachable devices such as syringes or pumps, for pipette-free, active control of sample actuation with volume metering [23]. Moreover, 3D-printed devices with on-chip reagent storage [14,24] can combine with and benefit from the capabilities of paper-based devices [25,26].

Here we present a multifunctional and miniaturized sample preparation unit that integrates with a consumer-operable prototype immunosensor for handheld, solid-liquid, multi-allergen extraction. The interconnectable ULOC then enables on-chip sample handling for equipment-free dilution, transport, and LFIA detection of hazelnut and peanut allergens in the low ppm range in spiked and commercial bakery products.

2. Materials & methods

2.1. Reagents and consumables

Multiplex LFIA and carbon nanoparticle labeled antibodies (CNP-mAbs) against hazelnut and peanut have previously been developed, characterized, and validated [13,27]. Running buffer (RB)/extraction buffer was 100 mM borate buffer (BB) pH 8.8, composed of 100 mM boric acid (Merck, Darmstadt; Germany) and 100 mM sodium tetraborate (VWR, Leuven; Belgium) with 1% (*w/v*) bovine serum albumin (BSA; Sigma-Aldrich, Zwijndrecht, The Netherlands) and 0.05% tween-20 (*v/v*) (Merck, Darmstadt; Germany). The 10 mL and 1 mL disposable plastic syringes that were used for the homogenizer and air displacement syringes were purchased from Becton-Dickinson (Utrecht, The Netherlands), and low binding syringe filters used to filter total protein extracts (5 μm; 1.2 μm; 0.45 μm) were acquired from Pall Life Sciences (Pall Netherlands B.V, Medemblik; The Netherlands). Silicon tubing for ULOC connectors was purchased from Esska-Tech (Arvika; Sweden). ULOCs were sealed on the open side with adhesive tape (3 M Ruban Adhesive Scotch Nastro Adhesive, 3 M Europe, Diegem; Belgium). Red food dye solution (consisting of water, propylene glycol, and Carmoisine CI 14720) of unknown concentration used for dilution characterization of the ULOC was purchased from a local supermarket. The clamp used for attaching the smartphone to the holder's frame was purchased from Wolfcraft (Wolfcraft, Kempenich; Germany).

2.2. Reference material preparation

Standardized certified reference materials for food allergens are not currently available; therefore, total hazelnut protein (THP), total peanut protein (TPP), and blank cookie (BC) extracts were prepared in-house [13,27]. See Supplementary Information (SI) Table S1 for ingredient lists and labeling information. Fresh protein aliquots were defrosted on the day of experiments, and the protein content was always checked before use by a NanoDrop (ND 3300, Isogen Life Sciences, De Meern; The Netherlands) protein analyzer. Different sample types were utilized to characterize each module of the prototype immunosensor (Table 1).

2.3. Development and fabrication

Free CAD software Autodesk Fusion 360 (Autodesk Inc. San Rafael, CA; USA) was used for designing 3D-printable parts and converting them to printable .stl files. Fig. 1 gives a schematic overview of the prototype platform; Fig. 2 provides an annotated photographic overview of the disassembled (2A) and assembled (2B) platform. The ULOC dilutor (Fig. 2C) was printed with a stereolithography (SLA) printer Form3 (FormLabs, Somerville, MA; USA) at 25 μm layer resolution using proprietary clear resin (Type O4, FormLabs). A fused deposition modeling (FDM) model (Hephestos 2, BQ, Madrid; Spain) was used to print the sieves (Supplementary Information (SI) Figure S1), device holder (SI Figure S2), and interchangeable LFIA cartridges (SI Figure S3).

2.3.1. Homogenizer

The handheld and interconnectable homogenizer unit enables total homogenization and solid-liquid protein extraction from solid food samples. 3D-printed sieves with approximate pore sizes of 0.5 mm were cut by laser (HL40-5g, Full Spectrum Laser LLC, Las Vegas, NV; USA) into discs (18 mm diameter; SI Figure S1). Two sieves (Fig. 1A) insert into a 10 mL syringe at an offset to each other. As the plunger pushes solid material against the first 3D-printed sieve, it breaks into smaller pieces which are subsequently blocked by the second sieve that is kept at an offset, preventing particles from blocking microchannels in the ULOC. Silicon tubing (1.5 mm inner diameter, 40 mm length) connects with the ULOC unibody connector. The tubing can be used as a finger pump or is connected by a second larger piece of silicon tubing (2.5 inner diameter, 20 mm length) to the syringe tip. The syringe pressure is then used for user-controlled actuation.

Table 1
Reference material and sample classification.

Sample type	Matrix	Spike	Concentration range	Used to Characterize
A	Water	Food Dye Solution	N/A	ULOC Dilutor
B	Blank cookie extract	Total hazelnut protein (THP) extract	1–1000 ppm (v/v)	LFIA performance
C	Blank cookie	Total hazelnut protein (THP) extract	1–1000 ppm (v/w)	Homogenizer
D1	Blank cookie	Hazelnut cookie	0.1–100 ppm (w/w)	Total prototype
D2	Blank cookie	Hazelnut cookie & peanut cookie	0.1–100 ppm (w/w)	Total prototype

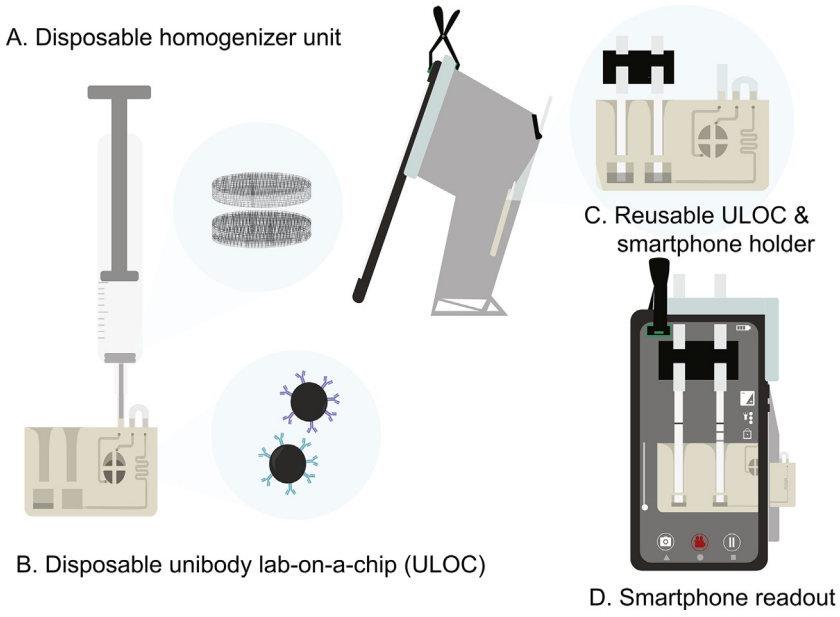

Fig. 1. Overview schematic of parts of the prototype immunosensor. (**A**) Disposable homogenizer unit with 3D-printed sieves (**B**) Disposable unibody lab-on-a-chip (ULOC) for dilution of extracted allergens and mixing with carbon nanoparticle labeled allergen-specific antibodies. (**C**) Reusable smartphone and ULOC holder. (**D**) Smartphone readout, as a result, appears in realtime on the screen.

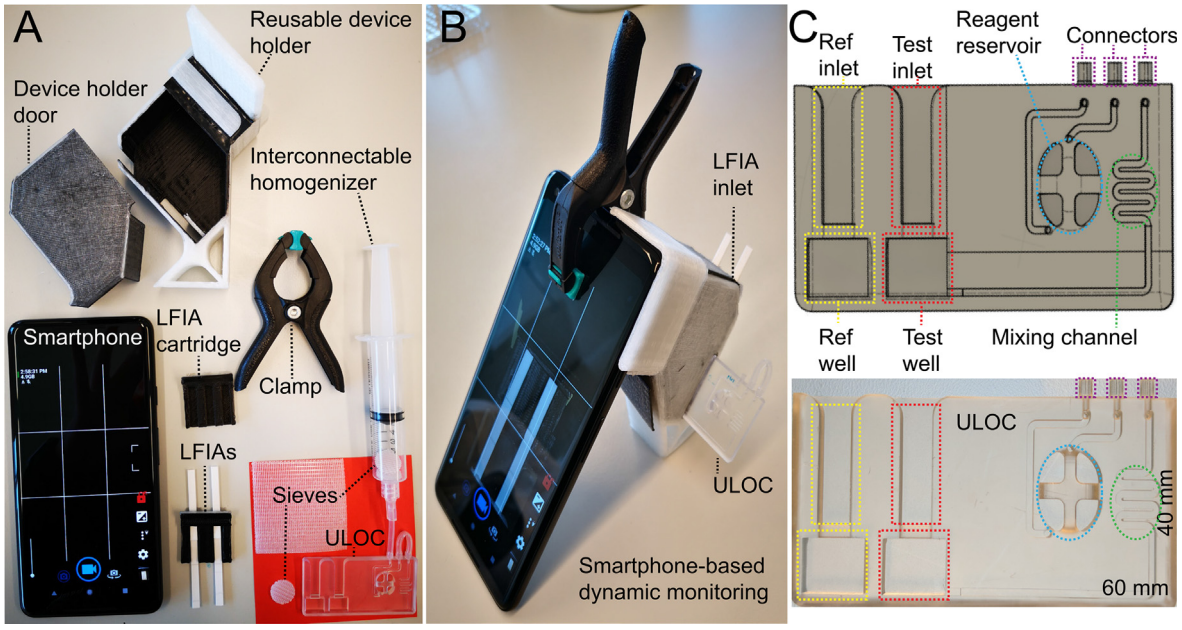

Fig. 2. Overview of prototype immunosensor. (**A**) Disassembled prototype immunosensor showing all components. (**B**) Side view of the assembled device where the Unibody Lab on a Chip (ULOC) slots into the smartphone holder, the smartphone clamps to the holder's frame, the LFIAs insert into the opening, and the results are viewed on the phone. (**C**) Annotated Computer-Aided Design (CAD) and photo of the ULOC.

2.3.2. ULOC dilutor

The ULOC dilutor (60 mm W x 40 mm L) has all functional features printed onto a single side. One side is left open, so uncured resin can be removed from 1 mm deep fluidic channels (1 mm wide) by sonicating (FinnSonic m15, FinnSonic Oy, Lahti, Finland) in ethanol (Sigma Aldrich, Steinheim, Germany) for 30 s and air drying. Before sealing the ULOC's open side with adhesive tape, CNP-mAbs and RB were pre-loaded into the reagent reservoir (volume capacity of 250 μL - for flexibility in adjusting RB volume, see Figure S4 for bioreagent loading areas) and the reference well (1 μL of CNP-mAb and 100 μL of RB) for control measurements. The RB both stabilized CNP-mAbs for on-chip storage and acted as a dilution buffer for injected samples. The test and reference wells were designed with an internal ledge to prevent any fluid overflow and had a total volume capacity of 200 μL each. Silicon tubing secured the first unibody connector with the homogenizer syringe tip. The remaining two unibody connectors were joined together by silicon tubing (see Fig. 1B).

2.3.3. ULOC & smartphone holder

The ULOC could be inserted into an opening (50 mm W x 35 mm L) in the 3D-printed device holder, which shielded the assay from ambient light (see SI; Figure S2). The LFIA cartridge, which fits 2 LFIAs (4 or 5 mm wide), ensured that the appropriate LFIAs were aligned with the test and reference wells in the ULOC. A smartphone was clamped to the outer frame of the 3D-printed holder overlaying the rear-facing camera and flash.

2.4. Characterization of prototype immunosensor

2.4.1. Extraction time

Pre-ground raw hazelnut was incubated in the homogenizer syringe with RB for different periods (1, 2, 3, 5, 10, 20, or 30 min) to optimize extraction time and assess 3D-printed sieve efficiency. The total protein concentration was quantified (n = 3) using the NanoDrop.

2.4.2. ULOC dilutor

Before characterization, 5 or 10 μL dye was actively injected via a disposable syringe into the manifold. Injections were repeated multiple times for distance verification, with the 5 and 10 μL distances being marked on the ULOC for convenience with subsequent sample loading (see SI Figure S5). The ULOC was characterized for its dilution ability by mixing dye with water (sample type **A**) at various dilution factors (DFs). Adjustable water volumes were pipetted into the ULOC reservoir. Dilution factors of x10, x15, x20, and x40 were achieved by injecting 5 or 10 μL of aqueous dye solution to the mark on the ULOC. For comparison with a manually pipetted sample, the same DF dye/water was pipetted into the ULOC reference well. Smartphone images of the ULOC were acquired using OpenCamera (v1.47.3) to keep exposure and focus constant on a Google Pixel 2 XL (Google, California; USA). On and off-chip dilutions were evaluated by comparing the color intensities in the test and reference wells at the end of the manifold [23]. Subsequently, images were processed offline using ImageJ [28] to split images into their RGB (red, green, blue) color channels. In the blue channel pixel intensity readings were taken from the test and reference wells for direct comparison.

2.5. Dynamic data acquisition

Images and videos were acquired by smartphone, attached to the holder frame, using OpenCamera to ensure fixed acquisition conditions (fixed focus, locked exposure, controlled illumination, for videos: 30 frames per second (fps), 720 × 480 pixels). LFIA results appeared on the screen as they emerged. Subsequently, videos were split into images of 1 fps using Adapter (v2.1.6), and the resulting time point images were analyzed offline in ImageJ by splitting the images into their color channels. A blue channel pixel intensity (BCPI) reading was taken from below the test line at t = 0 as a background response; the BCPI measurements from the test and control lines were then subtracted from this to give the corrected BCPI (cBCPI) value. In the assay dynamic working range, cBCPI increases as test and control lines increase in intensity. The T/C ratio is a standard metric used for normalizing sandwich format LFIA results [11] and has been applied here.

2.6. Prototype immunosensor characterization

Experiments were performed in triplicate; see Fig. 3 for the pictogram operation procedure and SI Video 1 for a short demo of the total system operation from extraction to LFIA readout. Before sealing the ULOC with adhesive tape, the reagent reservoir was filled with 2 μL CNP-mAb and 190 μL RB, resulting in a DF x 20 when 10 μL of sample was actively injected into the reservoir. Sample type **B** was used to characterize the LFIA immunochemistry and smartphone readout. Pre-weighed sample type **C** was used to evaluate the extraction and filtration by the homogenizer unit by comparing the LFIA result against results obtained with sample type **B**. To determine the size distribution of the resulting particles crushed by the homogenizer, we tested 3 different solid samples (i.e., BC, HC, and PC; n = 10) and took 3 individual aliquots and a pooled fraction for each cookie, photographed the particles, and analyzed their size distribution using ImageJ (see SI Protocol S1 for full details).

For sample types **C** and **D**, approximately 0.25 g of sample was incubated with 1 mL RB for 1 min, before filtering through the 3D-sieves. Finally, to characterize the system for detecting real-life incurred and processed allergens, sample type **D** was investigated. The volume of the pre-loaded CNP-mAb was increased to 4 μL (2 μL for the anti-hazelnut mAb-CNP and 2 μL for the anti-peanut mAb-CNP) for multiplex analysis. Here, we consistently injected the sample up to the 10 μL mark on the ULOC to assure the reproducibility of results. However, the sample injection is actively controlled by the user, and they can simply choose to inject the sample further into the ULOC if a greater volume/higher analyte concentration is required. Air displacement transported the sample to the detection well. The ULOC was then inserted into the device holder, the LFIAs inserted into the ULOC aligning with the test and reference wells, and the smartphone was set to video record to acquire the data. Here, the immunochromatographic limit of detection (LOD) was the lowest concentration at which two lines (test and control) can be visually, or by smartphone, distinguished compared to a blank sample (n = 3).

The supplementary video related to this article can be found at https://doi.org/10.1016/j.aca.2020.10.018

3. Results and discussion

3.1. Extraction time

A major restriction of allergen analysis is the lengthy extraction process, which typically includes weighing, heating, grinding, and numerous filtering steps [2,11]. As such, extended extractions delay rapid screening tests such as LFIA. While a 2 min magnet-assisted allergen antigen extraction has been reported, this still required off-chip microwave pre-heating [3]. Previously we described a method for extracting total proteins from cookies and peanut flour at room temperature (RT) in 30 min [13]. This method is promising because even with the shorter extraction time and at RT, the

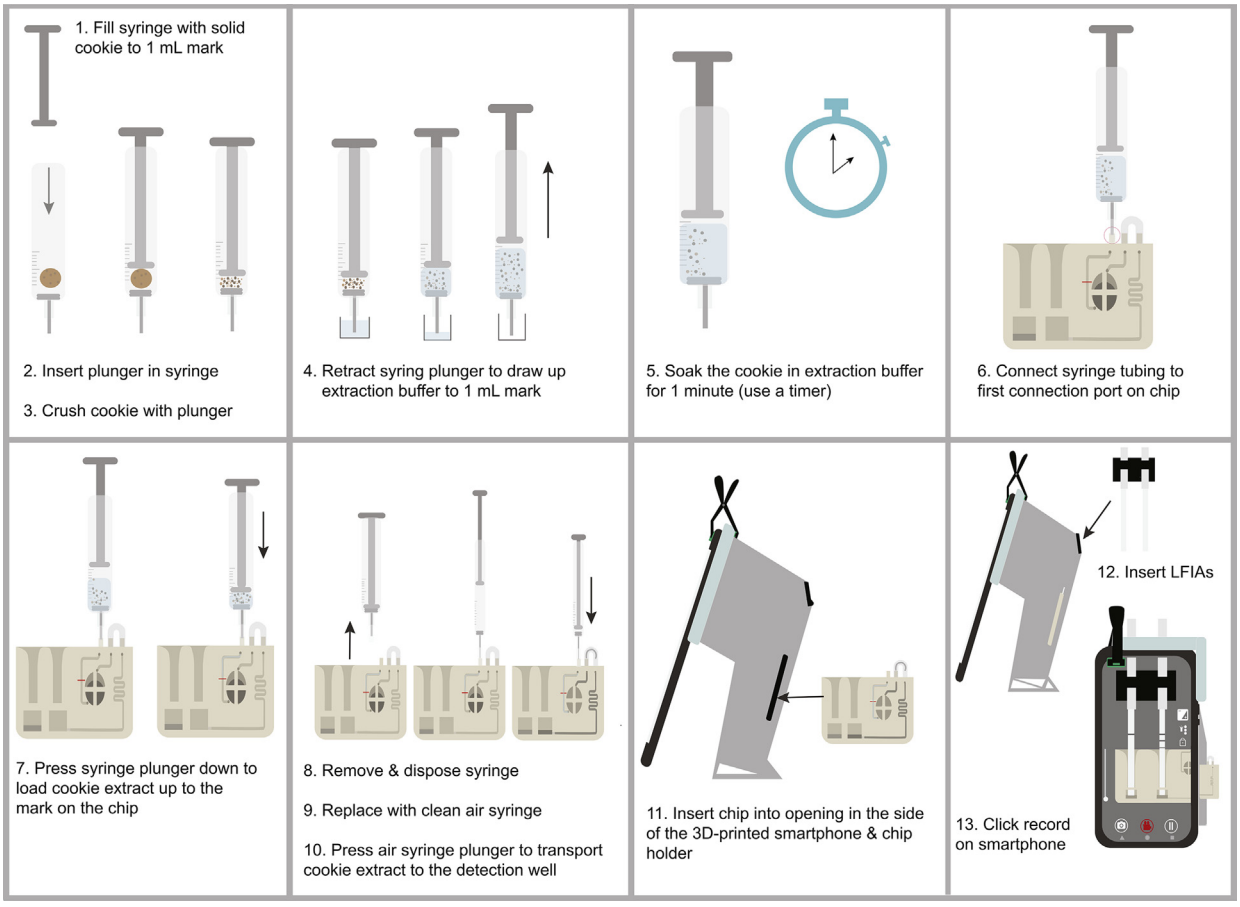

Fig. 3. Pictogram instructions for operating prototype immunosensor for homogenization, extraction, ULOC-based dilution, transport, and LFIA based detection of food allergens with on-screen smartphone readout. The guidelines have been designed to guide the consumer during citizen science experiments.

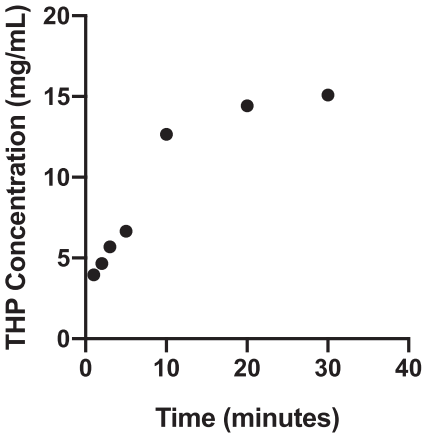

Fig. 4. Graph showing effects of different buffer incubation times (1, 2, 3, 5, 10, 20 & 30 min) on the total hazelnut protein (THP) concentration (mg/mL) in the extract from raw hazelnut (n = 3 extractions). Error bars are displayed but are too small to see; for standard deviation, see SI Table S2.

extracted samples still required extensive dilution to comply with the LFIA dynamic working range, indicating a much shorter extraction time could still be appropriate for extracting relevant allergenic proteins without delaying the analysis. To test this, we evaluated different extraction times to attempt to reduce the overall assay duration, see Fig. 4 and SI (Table S2).

High protein concentrations are extracted even within the first minute (RSD = 1.5%, $n = 3$), with the concentration increasing with longer buffer incubation time (first 10 min). The disposable 3D-sieves circumvented the need for further sample filtration, which typically is carried out in a stepwise fashion (see 2.1 & 2.2) to filter out lipids and larger particles. As well as filtering particles, crushing with the 3D-sieves and homogenizer led to reproducible particle size distribution (n = 10) between the 3 varieties of cookie (0.04–1.2 mm particle diameter) (see SI Figure S6). Unlike other reported integrated microdevices [3,10,14], our extraction requires no sample pre-treatment or heating; the detachable homogenizer unit interconnects with the ULOC, which then executes all outstanding sample handling. Only one other reported allergen screening device offers solid-liquid internal extraction in less than 4-min [1]. These experiments used pre-weighed samples, but to improve consumer-operability, the user can instead simply fill the homogenizer syringe with the solid sample to the 1 mL visual mark, avoiding the need for weighing equipment as this method gave reproducible sample weights ($n = 10$) for all 3 variations of cookie tested (see SI Figure S7). While this would not result in quantitative results, such an approach is adequate for semi-quantification. Currently, in this early prototype, the extraction buffer is provided in a pre-measured vial containing 1 mL. However, future refinement could include an additional ULOC chamber for on-chip extraction buffer storage.

3.2. ULOC dilutor

Allergenic proteins exist in foods over a broad dynamic range and must be detected at trace levels for protecting sensitive individuals. Still, it is vital to understand that highly concentrated samples can yield paradoxically low signal intensities, which could easily be misinterpreted by a consumer. However, it is reported that sample dilution (DF x 10–100) can minimize the occurrence of false-negatives [11–13]. While sample dilution is a prerequisite for allergen analysis, we cannot expect the consumer to do this. To circumvent the issue, we have created a system allowing for arbitrary, pipette-free, sample dilution by pre-storing adjustable volumes of RB in the reservoir. When the extracted sample is injected into the reservoir, it efficiently mixes with the pre-stored CNP-mAbs by air displacement and is also diluted in RB by an adjustable DF.

Fig. 5A compares the BCPIs for on-chip (ULOC-enabled) versus off-chip (manually pipetted) dilutions (dye in water, sample type **A**, n = 3) using different DFs. Fig. 5B indicates where to take the pre-dilution (DF x 0), the mid-dilution (5C), and the on-chip and off-chip (5D) BCPIs measurements. For consistency, the measurements were always taken below the dye's meniscus. The ULOC DFs invariably matched the manually pipetted DFs, suggesting that the ULOC delivers well-defined sample volumes (see SI Figure S5). ULOC devices for other applications have already been extensively characterized for integrated actuation (2–15 μL) with comparable accuracy to pipettes [23,25]. Injecting the sample causes turbulent mixing (Fig. 5C) because of the co-injection of air bubbles. Air metering for sample transport is also documented elsewhere [16]. The current combined immunosensor benefits from nitrocellulose. When the LFIA touches the turbid liquid, the nitrocellulose wicks the fluid, displacing the air from its pores, without bubbles disrupting the optical measurement.

3.3. Dynamic data acquisition

To investigate the influence of assay duration on the signal development, the LFIAs were readout after 5 (SI Figure [S8A]), 10 [S8B], 15 [S8C], and 20 [S8D] minutes with sample type **B** (0.1–1000 ppm). From these images, the test and control line signal development [S8E] and the T/C ratio development [S8F], are each plotted as a function of time (min) in independent calibration curves. At 5 min, the lowest concentration visibly readable is 10 ppm, with the signal improving with increased duration. After 10 min, a 1 ppm signal is readable. However, at 1000 ppm, no signal is generated on either line, even after 10 min. Even with ULOC-enabled sample dilution (DF x 20), high-concentration LFIA effects are observed, affirming the necessity of dilution to avoid false-negative results. The majority of LFIA developers recommend an assay duration of up to 20 min to allow the signal to reach full stability and sensitivity [8]. However, we found that a 15 min duration was suitable for allowing signals to develop for all concentrations, without neglecting more concentrated samples. In future versions, the ULOC could benefit from having multiple dilution wells for running 3 LFIAs simultaneously across an entire assay dynamic working range, further limiting the occurrence of concentration-dependent effects.

3.4. Total Allergen protein detection

Allergenic proteins can be subject to conformational alterations during food processing [29]. Therefore, biosensors must demonstrate proficiency in detecting allergens in both raw and processed food products. Here, solid cookie samples were pre-weighed (0.25 g) for consistency. Still, in real life, the user can instead fill the homogenizer with the cookie to the 1 mL mark to approximately obtain the same sample weight (see SI Figure S7). Though this method is less precise, it would suffice for qualitative assessment of bakery products for the presence of allergens. Previously, we found that extracted allergen samples still need extensive dilution before LFIA analysis [13]. Here, a manual DF x 20 (5 μL of THP in 95 μL RB) gave clear results at all tested concentrations (see SI Figure S9), without compromising detection at the lowest levels, so a DF x 20 was always applied for ULOC-enabled dilutions.

3.4.1. Total Allergen protein extract screening

See Fig. 6A for signal development under optimal conditions (e.g., THP extract spiked into RB). For sample **B** (THP extract spiked into BC extract (v/v); Fig. 6B), the T/C ratio detection limit is 1 ppm

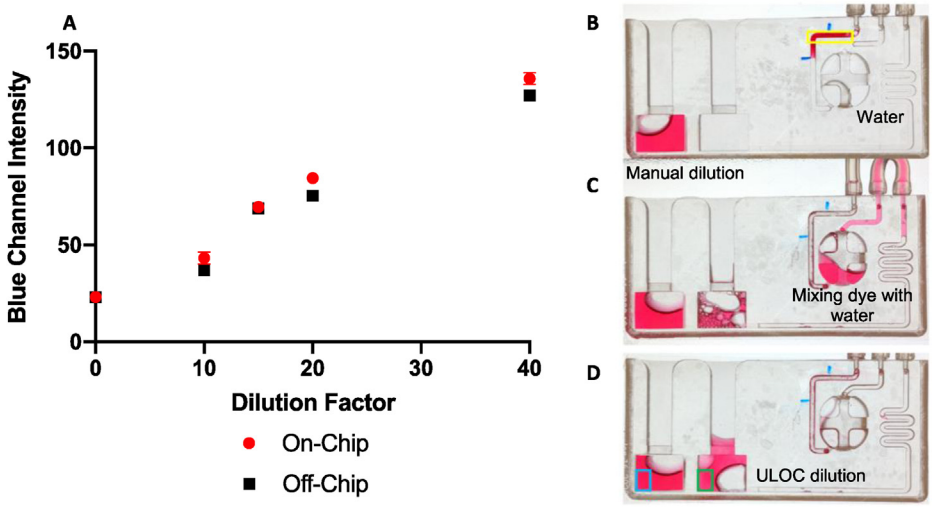

Fig. 5. ULOC dilutor. (**A**) Graph depicting the performance of ULOC for unidirectional sample dilution as a function of blue channel intensity. The red circle represents ULOC dilutions. The black square represents manual dilutions. (**B**) ULOC before dilution, the area where the volume metering reading is taken from is outlined in yellow. (**C**) ULOC during dilution, the dye mixes with water in the reagent reservoir and is delivered to the test area by the fluidic system. (**D**) ULOC after dilution, intensity reading for the manual dilution (outlined in blue), and the intensity reading for the ULOC dilution (outlined in green). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

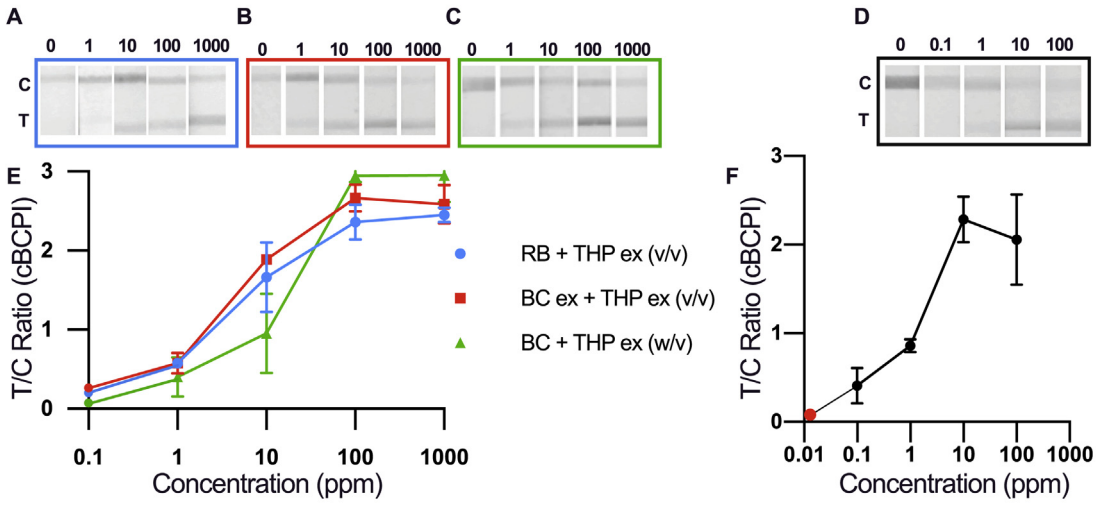

Fig. 6. Photographs and calibration curves showing LFIA signal development in increasing concentration of analyte where error bars represent standard deviation (n = 3) (A) 1–1000 ppm, total hazelnut protein (THP) extract spiked into running buffer (RB)(v/v); (B) 1–1000 ppm, THP extract spiked into a blank cookie (BC) extract (v/v); (C) 1–1000 ppm, BC spiked with THP extract (w/v); (D) 0.1–100 ppm, BC spiked with hazelnut cookie (HC) (w/w); (E) Calibration curve for [A; blue circle], [B; red square] and [C; green triangle] where circles represent the signal at 0 ppm; (F) Calibration curve for D, red circle represents 0 ppm measurement. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(n = 3). Despite using the prototype for analysis, the LOD here is not much higher than in our previous work (0.5 ppm; n = 20) [13], which was obtained using standardized laboratory conditions, pipettes, and equipment. Sample **B** measurements are reproducible (RSD ± 2.9%), indicating the ULOC mixes well and delivers persistent volumes, and that the LFIA still performs when combined with the ULOC. Solid sample type **C** (BC spiked with THP extract (w/v); Fig. 6C) was extracted and analyzed to reflect an actual solid-liquid extraction, with a LOD of 1 ppm (RSD at 1 ppm ± 3.7%). The slight increase in T/C deviation could be due to the crushing efficacy of the homogenizer. Small differences in buffer incubation times between repeat measurements and non-uniform dispersion of liquid THP extract could be consequential to the somewhat higher variation.

3.4.2. *Incurred single Allergen screening*

Thermal processing, such as baking, can affect allergen detectability [29]. See Fig. 6 to compare signal development for samples using total allergen protein extracts (6E) with samples containing incurred allergens (Fig. 6D&F). Testing commercial hazelnut cookies mixed with blank cookies (sample type **D1**) exemplifies the effectiveness of extracting incurred proteins from a solid matrix into a testable liquid and detecting the allergenic proteins in this liquid. Sample **D1** has a LOD of 0.1 ppm (n = 3, RSD ± 3.03%; see Fig. 6D and F) for processed hazelnut. Compellingly, the **D1** LOD is lower than the LOD for sample **C**. The LFIA is more sensitive towards processed hazelnut. This sensitivity has also been indicated in our singleplex hazelnut LFIA where the same mAb reached the same LOD for HC extract in BC extract [27].

In Fig. 6, high-concentration effects (1000 and 100 ppm) are evident. Even with the faster extraction time and ULOC-dilution concentration-dependent effects still occur, affirming the necessity to dilute allergen samples before analysis [11,12]. For consumer testing, the loss of the control line (at 1000 ppm) could be problematic, and some tests have additional target lines to limit this [1]. Here, we included a reference well in the ULOC pre-containing RB and CNP-mAb for a blank control. The consumer can then use this to directly compare the physical appearance of the test and control lines in realtime. Of course, in a dedicated smartphone-app, any human error would be avoidable, triggering an alert when the LFIA falls outside normality.

3.4.3. *Incurred multi-allergen screening*

Sample type **D2** (HC and PC in BC (w/w); Fig. 7) demonstrates the prototype's effectiveness for simultaneously co-extracting and

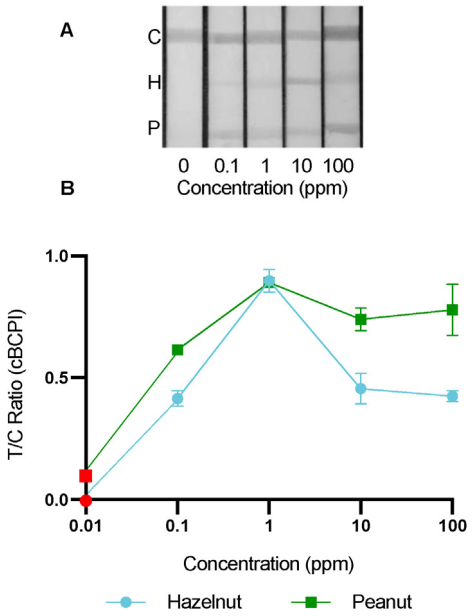

Fig. 7. Photographs and calibration curves showing multiplex LFIA signal development in increasing concentration of incurred multi-allergen analyte where error bars represent standard deviation (n = 3). (**A**) Multiplex calibration range for sample type **D2** (i.e., hazelnut cookie and peanut cookie spiked into blank cookie 0.1–100 ppm (w/w)) where C denotes the control line, H the hazelnut test line and P the peanut test line. (**B**) Calibration curve as a function of the T/C ratio (i.e., test line intensity divided by control line intensity) using corrected blue channel pixel intensities. The red circle represents the T/C ratio in a blank (0 ppm) sample. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

detecting unrelated processed allergens. Both analytes were detectable at 0.1 ppm (n = 3, RSD ± 2.5% and 1.6% for hazelnut and peanut, respectively). There is a slightly lower deviation in multiplex measurements owing to increased control line stability, from using two different CNP-mAbs compared with singleplex analysis. The sensitivity is even better than when using the same LFIAs to detect THP and TPP spiked into BC extract (v/v, LOD 0.5 ppm) [13] and has similar or higher sensitivity compared to other LFIAs [11,12].

3.5. Consumer diagnostics potential

The prototype was tested by an independent person with no scientific or technical background to demonstrate consumer-operability. According to the European Citizen Science Association (ECSA), citizen science should involve generating new knowledge that is beneficial to both the citizen and the researcher and with results being made public through open access publications [30]. We provided the participant with a blank and spiked cookie (sample type **C**) and two vials with pre-contained volumes of RB, 4 LFIAs, and the prototype immunosensor. Following a 5 min explanation and using the pictogram-based standard operating procedure (see Fig. 3), the participant performed the assay (n = 2; see SI Figure S10). He then placed the ULOC in the device holder, inserted the reference and test LFIAs, and recorded the result on the smartphone. The participant successfully differentiated between the positive and negative results for the spiked and blank samples, signifying the early prototype is already is operable by non-skilled individuals after only a short explanation.

4. Conclusions

The reported handheld immunosensor allows for interconnectable sample preparation, solid-liquid protein extraction, dilution, delivery, detection, and smartphone readout of multiple allergens in bakery products. The detachable homogenizer efficiently co-extracts and filters two major but distinct allergens from solid samples in record time. Active injection of the extracted liquid sample into the ULOC mixes the extract with RB for arbitrary sample dilution and with labeled bioreagents before delivery to the detection chamber. This pipette-free dilution limits the occurrence of false-negative results in LFIA. Realtime results are automatically readable as they develop on the phone screen. While the results are readable on the phone screen within 5 min, they are optimal after 15 min. The interchangeable LFIA cartridge means the reported system with ULOC-enabled sample dilution can easily be applied to test different LFIAs targeting various food, biomedical and forensic applications, affirming the value of such a simplified, adjustable, and multifunctional system.

The immunosensor is inexpensive, with current material costs of less than 1$/USD. The prototype is already consumer-operable, and further advancements, such as image processing in a dedicated smartphone app, will continually improve the usability of the system. The presented handheld system is an encouraging development for affordable, simplified multiplex consumer immunodiagnostics.

CRediT authorship contribution statement

Georgina M.S. Ross: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation. **Daniel Filippini:** Conceptualization, Methodology, Software, Writing - review & editing, Supervision. **Michel W.F. Nielen:** Resources, Writing - review & editing, Supervision, Funding acquisition, Project administration. **Gert IJ. Salentijn:** Conceptualization, Methodology, Writing - review & editing, Visualization, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank Thomas Tiemens for his participation in citizen science experiments. We would also like to thank Leo van Raamsdonk for making microscope photographs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.10.018.

Funding

This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement No. 720325.

References

[1] J. Zhang, S.B. Portela, J.B. Horrell, A. Leung, D.R. Weitmann, J.B. Artiuch, S.M. Wilson, M. Cipriani, L.K. Slakey, A.M. Burt, F.J. Dias Lourenco, M.S. Spinali, J.R. Ward, A. Seit-Nebi, S.E. Sundvor, S.N. Yates, An integrated, accurate, rapid, and economical handheld consumer gluten detector, Food Chem. 275 (2019) 446–456.
[2] A.S. Tsagkaris, J.L.D. Nelis, G.M.S. Ross, S. Jafari, J. Guercetti, K. Kopper, Y. Zhao, K. Rafferty, J.P. Salvador, D. Migliorelli, G.IJ. Salentijn, K. Campbell, M.P. Marco, C.T. Elliot, M.W.F. Nielen, J. Pulkrabova, J. Hajslova, Critical assessment of recent trends related to screening and confirmatory analytical methods for selected food contaminants and allergens, Trac. Trends Anal. Chem. 121 (2019) 115688.
[3] H.Y. Lin, C.H. Huang, J. Park, D. Pathania, C.M. Castro, A. Fasano, R. Weissleder, H. Lee, Integrated magneto-chemical sensor for on-site food allergen detection, ACS Nano 11 (2017) 10062–10069.
[4] G.M.S. Ross, M.G.E.G. Bremer, M.W.F. Nielen, Consumer-friendly food allergen detection: moving towards smartphone-based immunoassays, Anal. Bioanal. Chem. 410 (2018) 5353–5371.
[5] L. Szybist, Temporary Policy Regarding Certain Food Labeling Requirements during the COVID-19 Public Health Emergency: Minor Formulation Changes and Vending Machines, Guidance for Industry, United States, 2020 (U.S.) Department of Health and Human Services, Food and Drug Administration.
[6] J. Boxer, S. Weddell, D. Broomhead, C. Hogg, S. Johnson, Home pregnancy tests in the hands of the intended user, J. Immunoassay Immunochem. 40 (2019) 642–652.
[7] J.L.D. Nelis, A.S. Tsagkaris, M.J. Dillon, J. Hajslova, C.T. Elliott, Smartphone-based optical assays in the food safety field, Trac. Trends Anal. Chem. (2020) 115934.
[8] T.-T. Tsai, T.-H. Huang, N.Y.-J. Ho, Y.-P. Chen, C.-A. Chen, C.-F. Chen, Development of a multiplex and sensitive lateral flow immunoassay for the diagnosis of periprosthetic joint infection, Sci. Rep. 9 (2019) 15679.
[9] Y.K. Oh, H.-A. Joung, S. Kim, M.-G. Kim, Vertical flow immunoassay (VFA) biosensor for a rapid one-step immunoassay, Lab Chip 13 (2013) 768–772.
[10] M. Park, T.S. Seo, An integrated microfluidic device with solid-phase extraction and graphene oxide quantum dot array for highly sensitive and multiplex detection of trace metal ions, Biosens. Bioelectron. 126 (2019) 405–411.
[11] L. Anfossi, F. Di Nardo, A. Russo, S. Cavalera, C. Giovannoli, G. Spano, S. Baumgartner, K. Lauter, C. Baggiani, Silver and gold nanoparticles as multichromatic lateral flow assay probes for the detection of food allergens, Anal. Bioanal. Chem. (2018) 1905–1913.
[12] P. Galan-Malo, S. Pellicer, M.D. Pérez, L. Sánchez, P. Razquin, L. Mata, Development of a novel duplex lateral flow test for simultaneous detection of casein and β-lactoglobulin in food, Food Chem. 293 (2019) 41–48.
[13] G.M.S. Ross, G.IJ. Salentijn, M.W.F. Nielen, A critical comparison between flow-through and lateral flow immunoassay formats for visual and smartphone-based multiplex allergen detection, Biosensors 9 (2019).
[14] K.T.L. Trinh, T.N.D. Trinh, N.Y. Lee, Fully integrated and slidable paper-embedded plastic microdevice for point-of-care testing of multiple food-borne pathogens, Biosens. Bioelectron. 135 (2019) 120–128.

[15] J. Yin, Y. Suo, Z. Zou, J. Sun, S. Zhang, B. Wang, Y. Xu, D. Darland, J.X. Zhao, Y. Mu, Integrated microfluidic systems with sample preparation and nucleic acid amplification, Lab Chip 19 (2019) 2769–2785.
[16] K. Du, H. Cai, M. Park, T.A. Wall, M.A. Stott, K.J. Alfson, A. Griffiths, R. Carrion, J.L. Patterson, A.R. Hawkins, H. Schmidt, R.A. Mathies, Multiplexed efficient on-chip sample preparation and sensitive amplification-free detection of Ebola virus, Biosens. Bioelectron. 91 (2017) 489–496.
[17] C. Zhu, A. Hu, J. Cui, K. Yang, X. Zhu, Y. Liu, G. Deng, L. Zhu, A lab-on-a-chip device integrated DNA extraction and solid phase PCR array for the genotyping of high-risk HPV in clinical samples, Micromachines 10 (2019).
[18] G. Comina, A. Suska, D. Filippini, Low cost lab-on-a-chip prototyping with a consumer grade 3D printer, Lab Chip 14 (2014) 2978–2982.
[19] G.IJ. Salentijn, P.E. Oomen, M. Grajewski, E. Verpoorte, Fused deposition modeling 3D printing for (Bio)analytical device fabrication: procedures, materials, and applications, Anal. Chem. 89 (2017) 7053–7061.
[20] G. Comina, A. Suska, D. Filippini, 3D printed unibody lab-on-a-chip: features survey and check-valves integration, Micromachines 6 (2015) 437–451.
[21] M. Zeraatkar, D. Filippini, G. Percoco, On the impact of the fabrication method on the performance of 3D printed mixers, Micromachines 10 (2019).
[22] J. Park, J.-K. Park, Integrated microfluidic pumps and valves operated by finger actuation, Lab Chip 19 (2019) 2973–2977.
[23] A. Suska, D. Filippini, Autonomous lab-on-a-chip generic architecture for disposables with integrated actuation, Sci. Rep. 9 (2019) 20320.
[24] J. Reboud, G. Xu, A. Garrett, M. Adriko, Z. Yang, E.M. Tukahebwa, C. Rowell, J.M. Cooper, Paper-based microfluidics for DNA diagnostics of malaria in low resource underserved rural communities, Proc. Natl. Acad. Sci. U. S. A. 116 (2019) 4834–4842.
[25] A.S. Tsagkaris, J. Pulkrabova, J. Hajslova, D. Filippini, A hybrid lab-on-a-chip injector system for autonomous carbofuran screening, Sensors 19 (2019) 5579.
[26] G.IJ. Salentijn, H.P. Permentier, E. Verpoorte, 3D-Printed paper spray ionization cartridge with fast wetting and continuous solvent supply features, Anal. Chem. 86 (2014) 11657–11665.
[27] G.M.S. Ross, M. Bremer, J.H. Wichers, A. van Amerongen, M.W.F. Nielen, Rapid Antibody Selection Using Surface Plasmon Resonance for High-Speed and Sensitive Hazelnut Lateral Flow Prototypes, in: Biosensors (Basel), vol. 8, 2018.
[28] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, Nat. Methods 9 (2012) 671–675.
[29] T. Rahaman, T. Vasiljevic, L. Ramchandran, Effect of processing on conformational changes of food proteins related to allergenicity, Trends Food Sci. Technol. 49 (2016) 24–34.
[30] ECSA, Ten Principles of Citizen Science European Citizen Science Association, 2020. https://ecsa.citizen-science.net/.