Detection of adulteration with cow milk of milk from other species through an immersible photonic immunosensor

D. Kourti[a], M. Angelopoulou[a], K. Misiakos[b], E. Makarona[b],

A. Economou[c], P. Petrou[a] and S. Kakabakos[a]

[a] *Immunoassays/Immunosensors Lab, Institute of Nuclear &Radiological Sciences & Technology, Energy & Safety, NCSR "Demokritos", Aghia Paraskevi GR-15341, Greece*

[b] *Institute of Nanoscience & Nanotechnology, NCSR "Demokritos", Aghia Paraskevi GR-15341, Greece*

[c] *Analytical Chemistry Lab, Department of Chemistry, National and Kapodistrian University of Athens, Panepistimiopolis Zografou GR-15771, Greece*

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Milk adulteration

Cow milk: **>20 allergenic proteins**

Caseins and beta-lactoglobulin are shown to cause the most allergenic effects.

Ewe and goat milk:
- ✓ less allergenic compared to cow milk
- ✓ higher nutritional value compared to cow milk
- ✓ seasonal availability
- ✓ higher price

Attractive for adulteration with cow milk.

- The quality and authenticity of milk are of paramount importance for both **consumers** and **dairy industry**.

- The maximum acceptable content of cow milk in milk from other species set by the European commission (Directives 2000/13/EC and 2003/89/EC) is **1% (w/w)**.

Detection methods for milk adulteration

- **Enzyme-linked immunosorbent assays (ELISA)**
- **Polymerase chain reaction (PCR)**
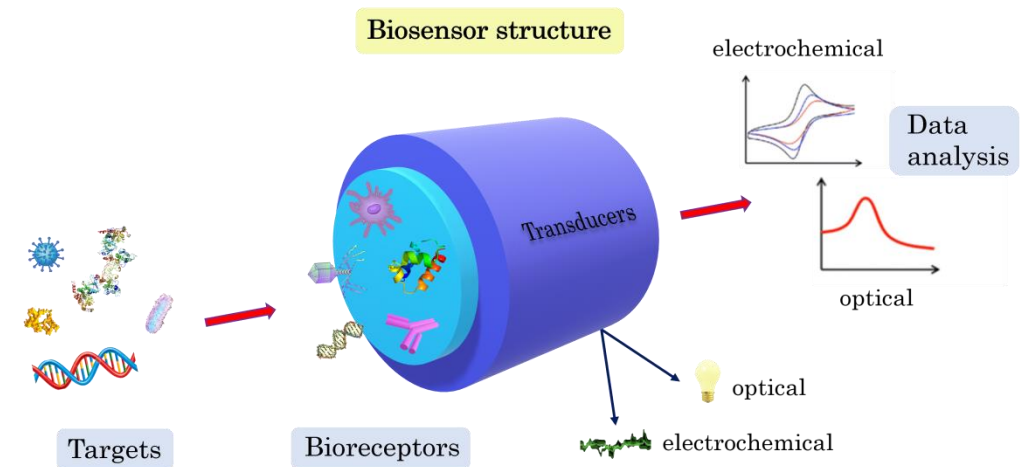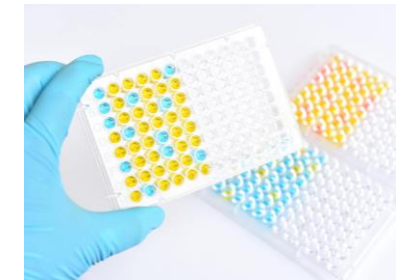- **High-Performance Liquid Chromatography (HPLC)**

  Disadvantages
  - ✓ expensive and bulky instrumentation
  - ✓ trained personnel
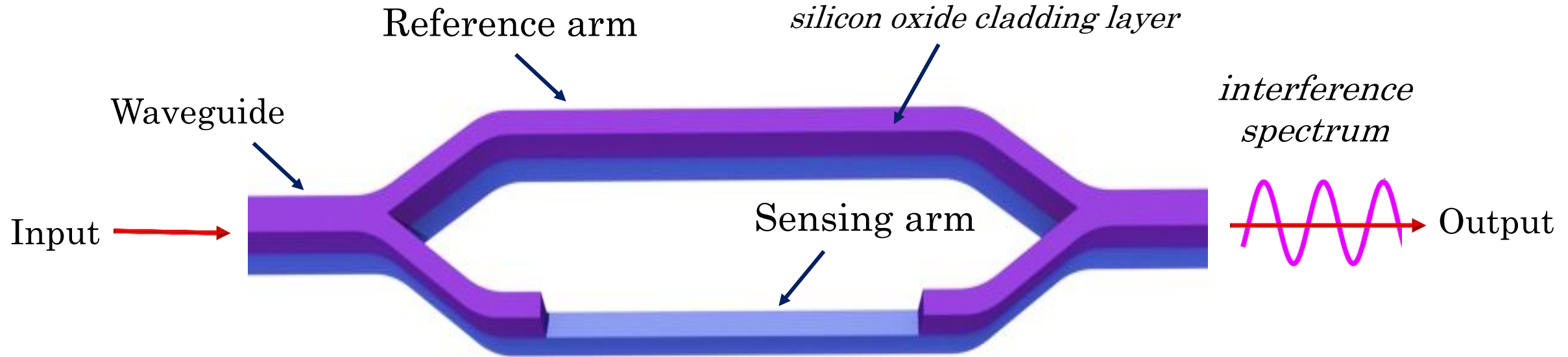 - ✓ not suitable for point-of-need applications

- **Biosensors**

  Advantages
  - ✓ Shorter assay time
  - ✓ Lower cost of analysis
  - ✓ Potential for multi-analyte determinations
  - ✓ Quantitative results in real-time
  - ✓ Potential for fabrication of portable devices

Reference arm

silicon oxide cladding layer

Waveguide

interference spectrum

Input

Sensing arm

Output

In an **integrated MZI**, a planar waveguide is split into two arms, the **sensing** and the **reference arm**, by means of a Y-junction. The MZI is covered by a silicon oxide cladding layer that has been removed from a certain long area over the sensing arm to allow for interaction of waveguided photons with sample. Due to the opening in the sensing arm, when the two arms combine again, an **interference spectrum** is created. Biomolecular reactions on the sensing arm, change its **refractive index** causing a **phase shift** of the interference spectrum and thus providing a way to monitor the adlayer growth on the sensing arm.
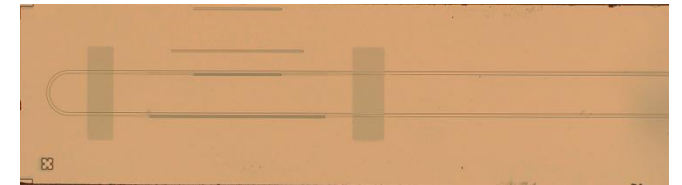
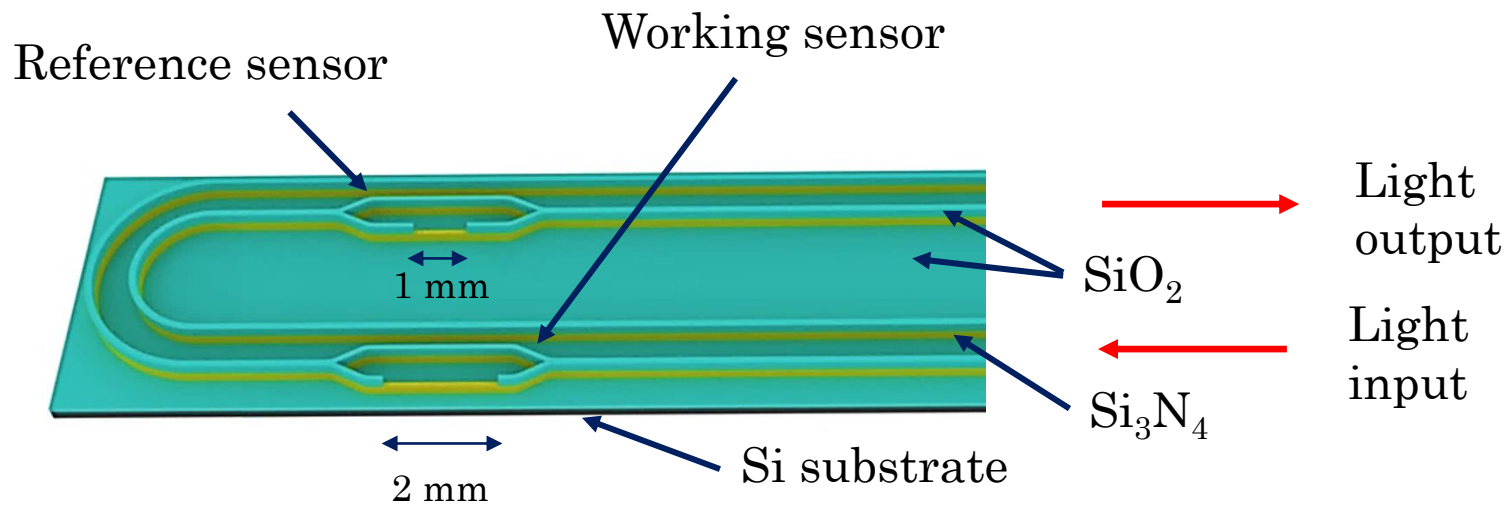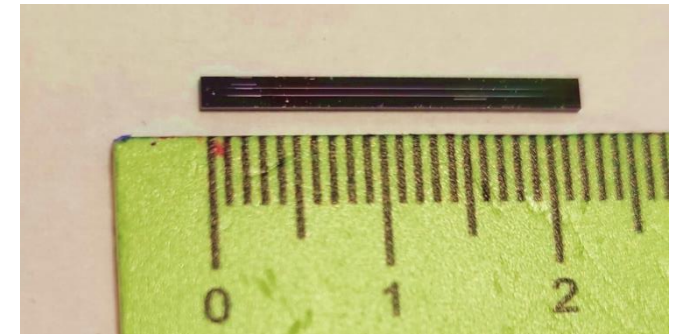# Broad Band Mach-Zehnder Interferometer on silicon chip

- ✓ Immersible chip
- ✓ Consisted of two U-shaped silicon nitride waveguides based on Mach-Zehnder interferometry
- ✓ The two U-shaped silicon nitride waveguides allow light in- and out-coupling from the same chip side
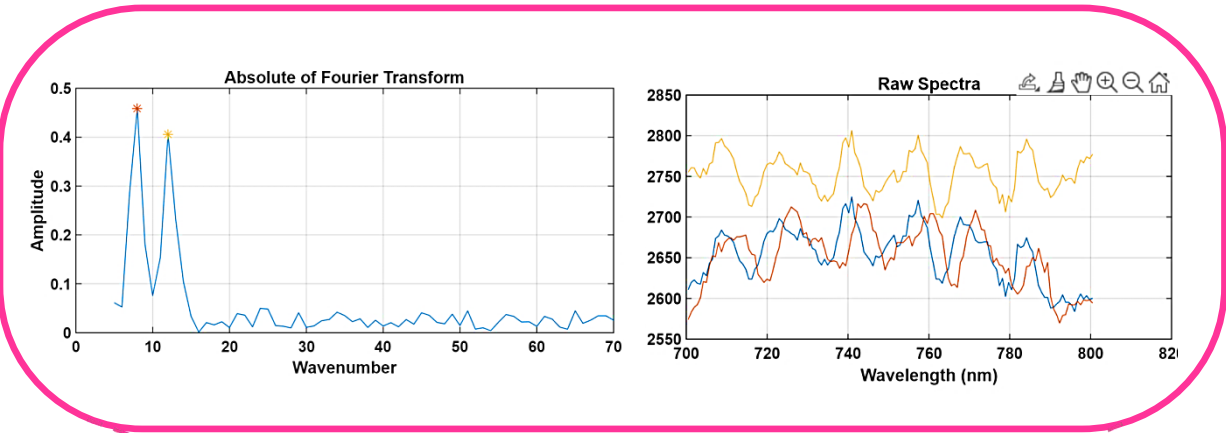
Image from optical microscope:

Actual size of the chip:

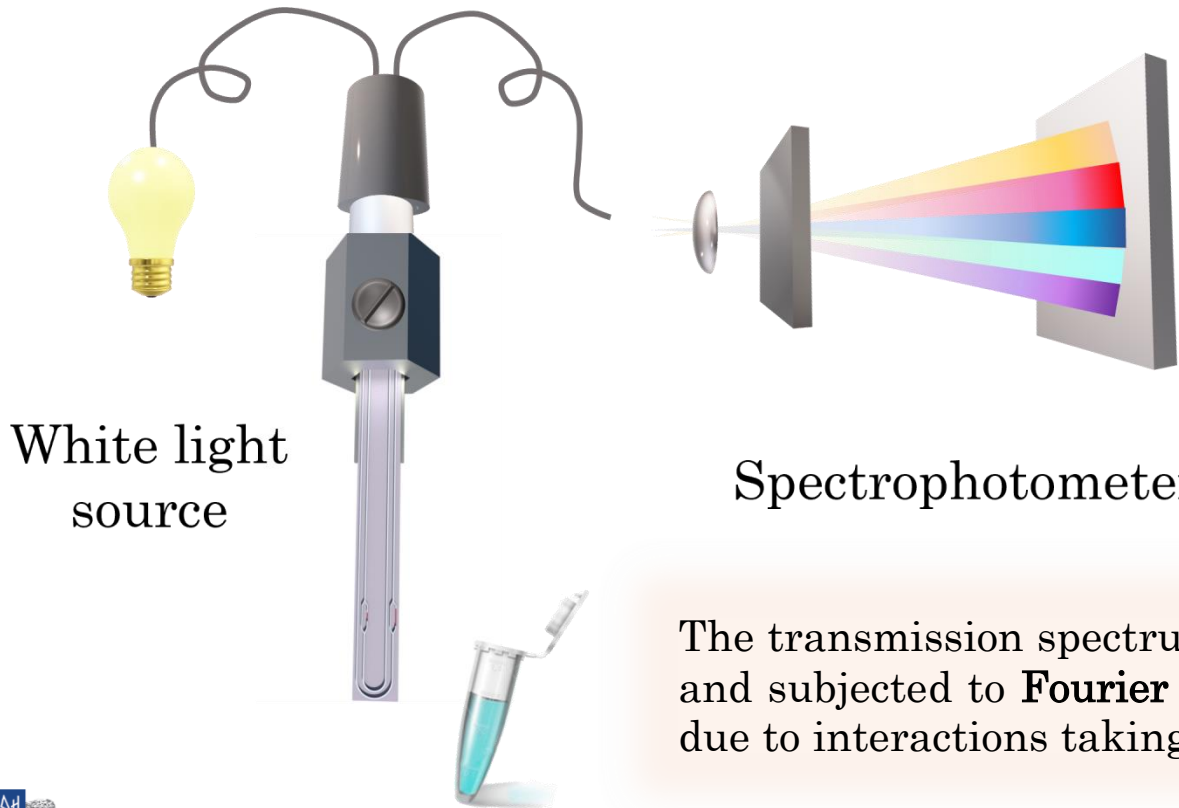Reference sensor

Working sensor

1 mm

2 mm

SiO_2

Si_3N_4

Si substrate

Light output

Light input

The chips are connected to a broad-band white light source and an external spectrophotometer through a bifurcated optical fiber.

White light source

Spectrophotometer

Fast Fourier Transform

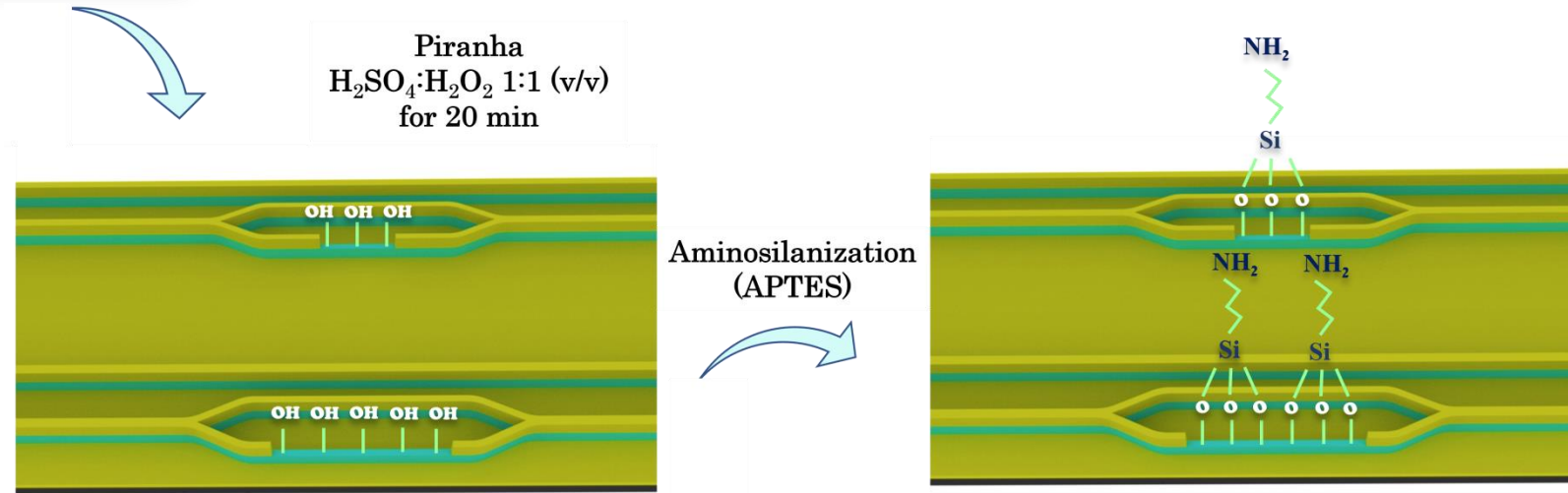**Real-time monitoring**

The transmission spectrum of both MZIs is recorded continuously during the assay and subjected to **Fourier Transform** to distinguish the phase shift of the two MZIs due to interactions taking place on them and provide for real-time monitoring.
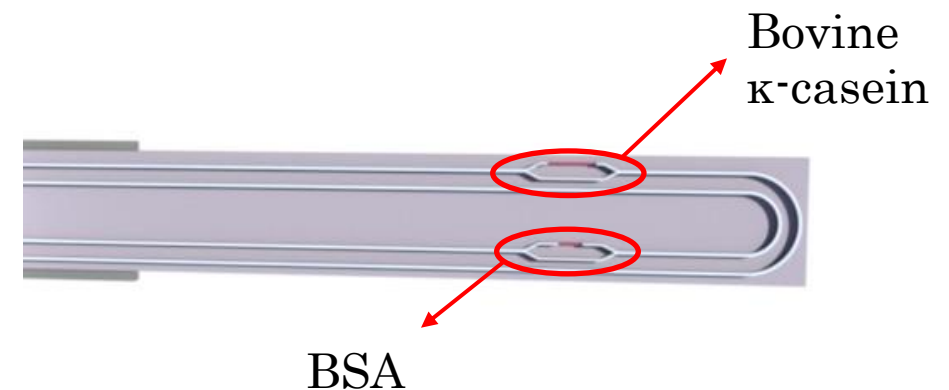
In order to transform the MZIs to biosensing elements, chemical activation was required in order to achieve stronger adhesion of the biomolecules to the surface.

Piranha
H_2SO_4:H_2O_2 1:1 (v/v)
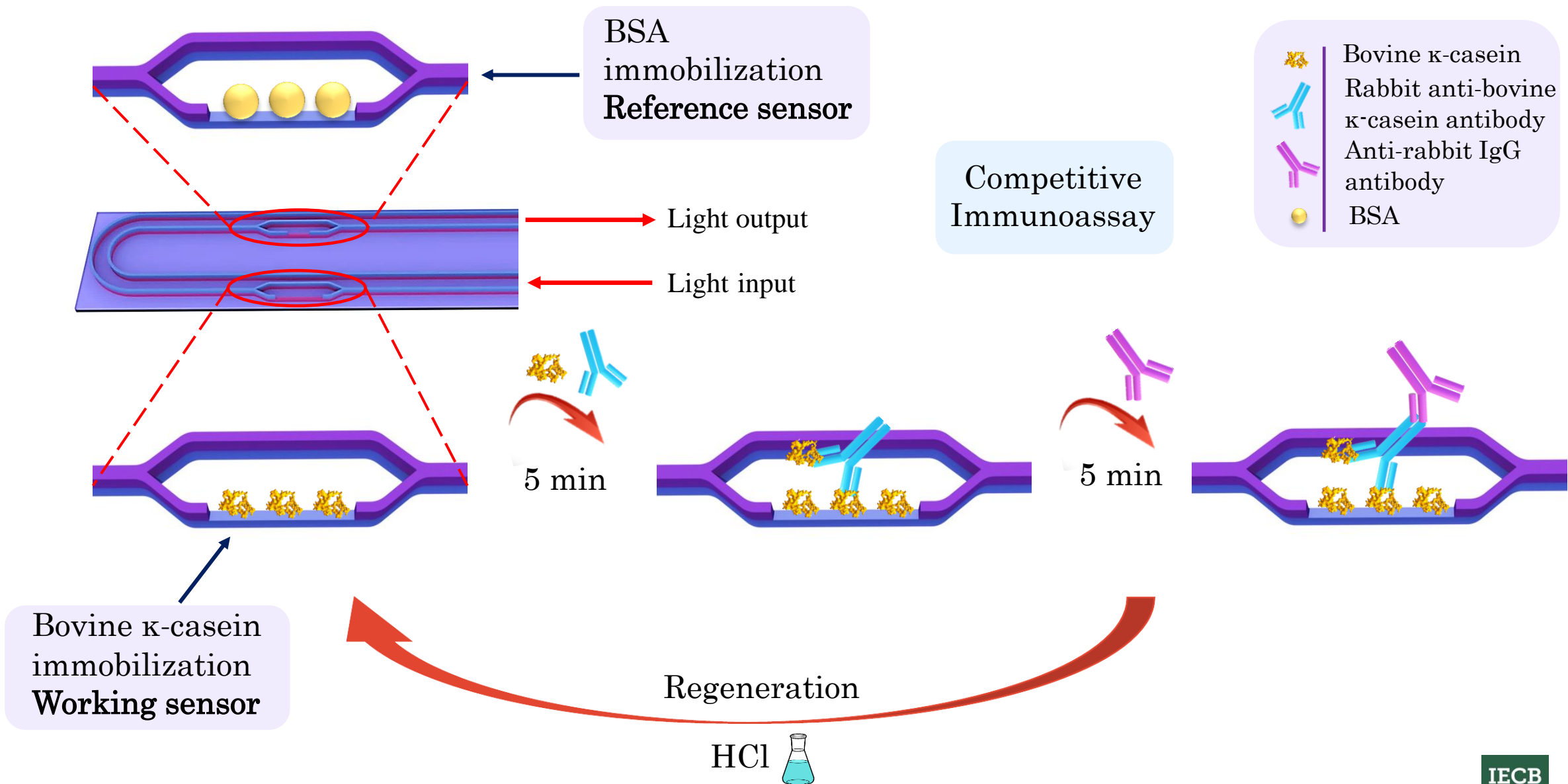for 20 min

OH OH OH

OH OH OH OH OH

Aminosilanization
(APTES)

NH_2

Si

NH_2 NH_2

Si   Si

Biological Functionalization

The biological activation of the chip was performed using a microarray spotter.

Bovine
κ-casein

BSA

Immunochemical reaction

BSA immobilization
Reference sensor

Light output

Light input

Competitive Immunoassay

Bovine κ-casein
Rabbit anti-bovine κ-casein antibody
Anti-rabbit IgG antibody
BSA

5 min

5 min

Bovine κ-casein immobilization
Working sensor

Regeneration

HCl

In order to develop the immunoassay for ewe/goat milk adulteration with cow milk, several parameters were optimized. It was found that:
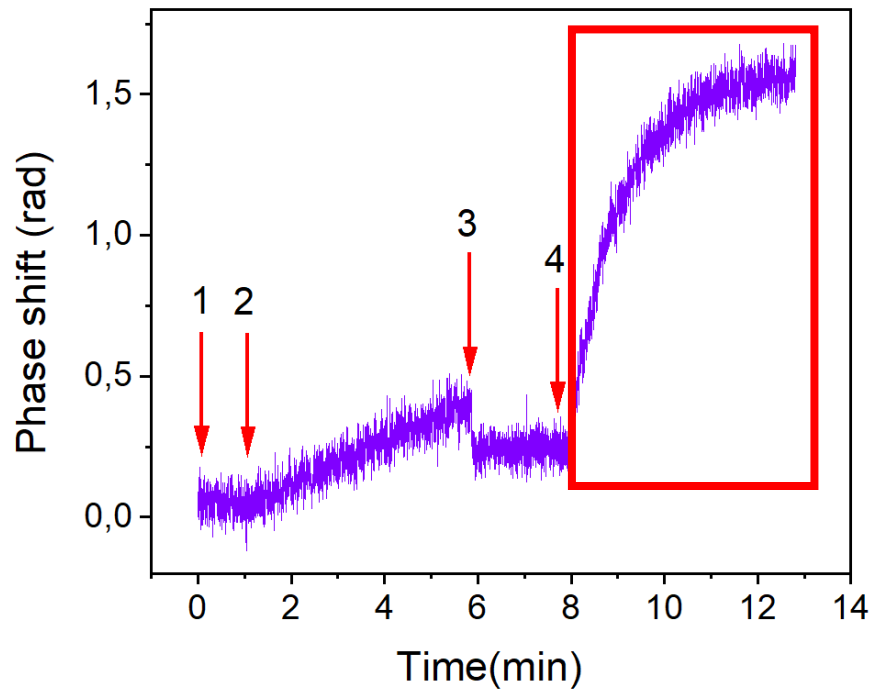
✓ Adequate signal response, along with high sensitivity was achieved when the bovine κ-casein concentration used for spotting of the chip surface was equal to or higher than **50 μg/mL**, employing 50 times anti-k-casein antiserum dilution.

✓ **1 h preincubation time** significantly increased the assay detection sensitivity.

Regarding the matrix effect, it was found that calibration curves obtained using bovine κ-casein calibrators prepared in 50 times diluted ewe/goat milk or assay buffer were identical.
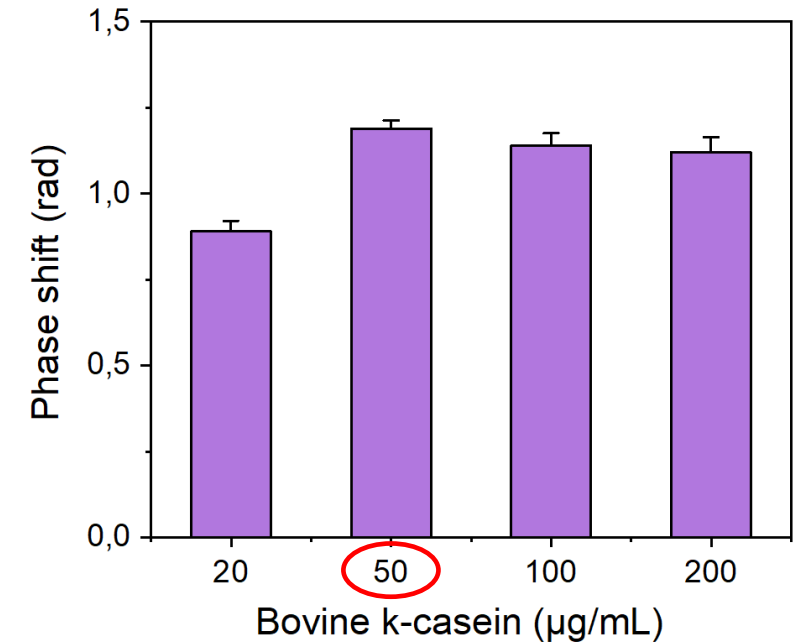
Signal responses obtained for zero calibrator in assay buffer when different bovine κ-casein concentrations were used for immobilization on the sensing arm of the working sensor.

Real-time **net signal** evolution of the zero calibrator prepared in 50-time diluted ewe milk

✓ The **net signal** is determined by the difference of the signal obtained from the working sensor minus that of the reference sensor.

✓ **The analytical signal** used is the phase difference obtained during the reaction with the anti-rabbit IgG antibody.

1 to 2	➡	1:100 ewe milk in assay buffer
2 to 3	➡	mixtures of rabbit polyclonal anti-κ-casein antiserum with calibrators prepared in 50-time diluted milk
3 to 4	➡	1:100 ewe milk in assay buffer
4	➡	anti-rabbit IgG antibody prepared in 100-time diluted milk

Typical calibration curves of bovine κ-casein in assay buffer and ewe milk
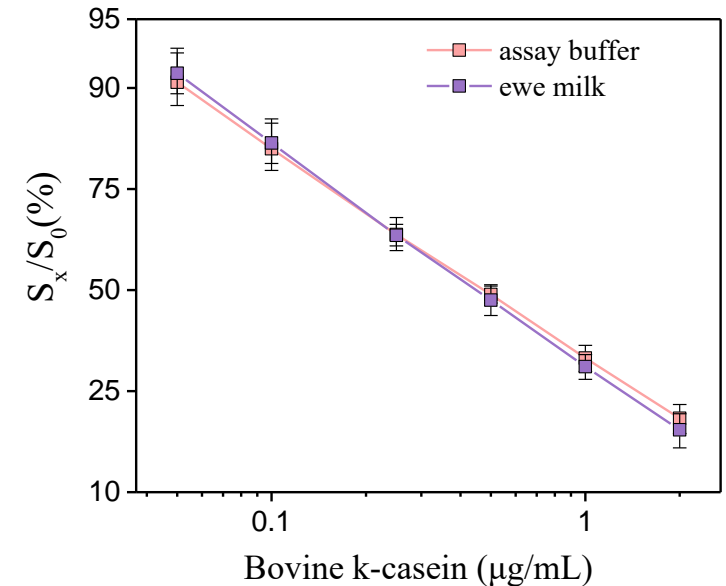
Detection limit	0.04 µg/mL
Dynamic range	0.1-2 µg/mL
Intra-assay CV	<4%
Inter-assay CV	<6.5%
Total assay time	12 min

An immersible photonic immunosensor based on Mach-Zehnder interferometry, that does not require external pumps and microfluidics, and thus simplifying the instrumentation and the assay procedure is demonstrated for the first time.

✓ The assay developed was sensitive with a LOD of **0.04 μg/mL** bovine κ-casein, with a working range from 0.1 to 2 μg/mL.

✓ Based on the mean content of bovine milk in κ-casein (3.4 g/L), it is calculated that the lowest detectable amount of cow milk in undiluted ewe or goat milk that can be detected with the method developed is less than 0.058%, which is well below the EU limit (1%) regarding adulteration of ewe/goat milk with cow milk.

✓ The assay was repeatable with intra- and inter- assay coefficient of variations lower than 4 and 6.5%.

✓ The total analysis time was 12 min.

✓ The excellent analytical characteristics and the small instrument size make the developed sensor ideal for on-site detection of milk adulteration.

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THANK YOU

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