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RESEARCH PAPER

A new analytical platform based on field-flow fractionation and olfactory sensor to improve the detection of viable and non-viable bacteria in food

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Abstract An integrated sensing system is presented for the first time, where a metal oxide semiconductor sensor-based electronic olfactory system (MOS array), employed for pathogen bacteria identification based on their volatile organic compound (VOC) characterisation, is assisted by a preliminary separative technique based on gravitational field-flow fractionation (GrFFF). In the integrated system, a preliminary step using GrFFF fractionation of a complex sample provided bacteria-enriched fractions readily available for subsequent MOS array analysis. The MOS array signals were then analysed employing a chemometric approach using principal components analysis (PCA) for a first-data exploration, followed by linear discriminant analysis (LDA) as a classification tool, using the PCA scores as input variables. The ability of the GrFFF-MOS system to distinguish between viable and non-viable cells of the same strain was demonstrated for the first time, yielding 100 % ability of correct prediction. The integrated system was also applied as a proof of concept for multianalyte purposes, for the detection of two bacterial strains (*Escherichia coli* O157:H7 and *Yersinia enterocolitica*) simultaneously present in artificially contaminated milk samples, obtaining a 100 % ability of correct prediction. Acquired results show that GrFFF band slicing before MOS array analysis can significantly increase reliability and reproducibility of pathogen bacteria identification based on their VOC production, simplifying the analytical procedure and largely eliminating sample matrix effects. The developed GrFFF-MOS integrated system can be considered a simple straightforward approach for pathogen bacteria identification directly from their food matrix.

Keywords Electronic olfactory system · Field-flow fractionation · Metal oxide semiconductor (MOS) sensor array · Pathogen bacteria · Viable and non-viable bacteria · Chemometrics

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Introduction

Contamination of foodstuffs with pathogen bacteria is a relevant and continuously growing global public health issue in developed and even more in developing countries. Sensitive, rapid, cost-effective and high-throughput analytical methods are required to enable large-scale screening procedures along the entire food chain, thus significantly improving food safety at reasonable costs.

Culture-based and biochemical identification methods are the gold standard, but they suffer from long assay times (up to 7–10 days, depending on the microorganism) and relatively high costs (a well-equipped laboratory and skilled manpower

are required), thus demanding for new rapid analytical methods.

Several alternative methods have been proposed in the last decades, including immunoassays, polymerase chain reaction (PCR)-based assays, DNA microarrays, biosensors and miniaturised microfluidic-based devices [1–3]. The ideal assay should enable direct analysis of the food sample, significantly reduce assay time and costs, still maintaining the high detectability and reliability offered by culture-based methods. A largely unsolved issue for most methods is the possibility to distinguish between viable and non-viable bacterial cells present in a sample, which would enable assessment of the real pathogenic potential of the food item and of the ability of food-processing treatments to kill pathogen bacteria. Few analytical methods can selectively detect living bacterial cells, such as PCR procedures coupled with an ethidium monoazide bromide pre-analytical treatment [4–6], PCR amplification of bacterial RNA [7] or Fourier-transform infrared spectroscopy (FTIR) [8].

New bioanalytical methods are often based on the availability of specific probes that recognise targets (e.g. proteins, nucleic acid sequences) characteristic of the bacteria population of interest. An alternative and more versatile system can be represented by fingerprint approaches able to give a complex instrumental pattern for each specific bacteria population, assisted by chemometric analysis to obtain qualitative and quantitative results. These approaches, which are fast, simple and not expensive, are usually based on proteomic mass spectrometry (MS) analysis, FTIR, light scattering or electronic noses. In a previous paper, we demonstrated the identification of bacteria on the basis of their characteristic protein mass spectrum fingerprint acquired by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) [9].

A metabolome approach has also been proposed, based on the detection of the specific pattern of volatile organic compounds (VOCs) produced by a given bacterial population. It is indeed well documented that the pattern of VOCs produced by microorganisms is highly related to the strain metabolome and thus species specific and also dependent on the growth phase (e.g. exponential, stationary) and conditions [10, 11].

The electronic nose comprises an array of olfactive sensors (most commonly metal oxide semiconductor (MOS), conducting polymer (CP) or surface acoustic wave (SAW) sensors) characterised by different chemical selectivities [12]. By subjecting the sensor signals to a suitable pattern analysis, a digital olfactory fingerprint is obtained for each sample and unknown samples can be classified upon preliminary training process [13]. Various authors proposed the use of electronic nose for early and rapid detection of bacterial contamination in food or consequent food spoilage, based on the analysis of the specific pattern of VOCs [14–18]. Employing an array of metal oxide-silicon field-effect transistors (MOSFET), we have previously shown that this technology can also detect changes in the VOC composition of the headspace due to metabolic changes induced on cells by a chemical compound [15]. Despite the advantages of these approaches, their diffusion is limited since they suffer from low reproducibility and robustness in particular for the analysis of complex samples. In addition, the detection of only viable bacteria is still an open issue.

All these aspects suggest combining a separation procedure as pre-analytical step with detection methods to increase the selectivity of the assay, providing rapid methods acting directly on the sample.

In this contest, field-flow fractionation (FFF) is a family of techniques suitable for the separation of high molecular weight analytes in complex matrices. FFFs are extensively used in hyphenation with different techniques for the development of multiparameter analytical platform, such as on-line coupling with mass spectrometry for proteomic analysis or on-line coupling with fluorescence and light scattering for conformational and dimensional analysis for quality control of nanoparticles material [19]. Among FFF, the gravitational field-fractionation variant (GrFFF) was already demonstrated able to fractionate cells from a complex matrix, based on their morphological characteristics [20, 21]. Cell separation is performed within an empty plastic channel due to the combination of two orthogonal forces: a laminar flow of mobile phase along the channel and a perpendicular field due to Earth gravity. Based on their size, shape, density and surface properties, analytes are positioned at different heights within the channel, where they encounter a fluid layer travelling at a given velocity. Analytes travelling towards the centre of the channel are thus eluted faster than those travelling close to the channel walls.

Due to its ‘soft’ separation mechanism, biocompatibility, possibility of employment in sterile conditions, GrFFF has proven to be very useful in the fractionation of bacteria [22–28] without perturbing their vitality and growth ability. Due to the simplicity of use and ancillary instrumentation required, we have previously demonstrated the possibility to on-line couple GrFFF with other orthogonal detection techniques with significant improvements of analytical performance [29–34]. In addition, any composition of mobile phase can be chosen and analytes can be injected directly from their matrix.

Herein, we present an integrated sensing system (GrFFF-MOS), in which an electronic olfactory system comprising an array of six MOS sensors (MOS array) is directly coupled to a GrFFF module to prepare enriched fractions of viable cells from complex matrices for the analysis.

The developed GrFFF-MOS sensing system was employed for the direct analysis of milk samples artificially contaminated with bacteria. *Escherichia coli* O157:H7 and/or *Yersinia enterocolitica* were used as sample models. Samples were

injected in the GrFFF module and then collected fractions, purified from sample matrix components, were directly subjected to MOS array analysis. The possibility to distinguish between viable and non-viable cells of the same strain was demonstrated. The MOS array signals were analysed employing a chemometric approach using principal components analysis (PCA) for a first data exploration, followed by linear discriminant analysis (LDA) as a classification tool, using the PCA scores as input variables. In addition, the ability of GrFFF-MOS to independently detect two bacterial strains simultaneously present in a milk sample was shown.

Materials and methods

Samples

The bacterial strains *E. coli* O157:H7 (ATCC 35150) and *Y. enterocolitica* (ATCC 23716) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). For cultivation, bacterial cells from a single colony grown on Luria-Bertani (LB) agar were transferred in 5–7 mL of LB broth (Bacto® LB broth, LENNOX; DIFCO Laboratories; Detroit, MI) and were grown for 15–18 h at 37 °C under shaking. In order to prepare non-viable bacteria samples, cell suspension in LB broth were heated for 30 min at 70 °C [35, 36]. The inactivation of heat-treated bacteria was confirmed by streaking 1 mL of cells suspension on LB agar and observing no bacteria growth upon incubation at 37 °C for 24 h.

Bacterial samples (either a single bacterial population or binary mixtures) were prepared either in LB broth or in skimmed milk and they were directly analysed with the GrFFF-MOS system. Each sample had a total bacteria concentration of 2.4×10^9 CFU mL^{-1} (as evaluated by optical density measurement at 600 nm), from which the concentration of each bacterial cell population in binary mixtures can be calculated (e.g. 1.2×10^9 CFU mL^{-1} each, in 1:1 binary mixtures).

Instrumentation and sample analysis

The analytical platform proposed includes the integration of a separative GrFFF compact tool with a MOS array sensing module. The proposed configuration was demonstrated suitable for the direct analysis of complex samples and able to give rapid and accurate results for the bacteria identification.

A scheme of the GrFFF-MOS system is reported in Fig. 1.

GrFFF module

The GrFFF device employed in this study was derived from a design already applied to the fractionation of bacterial cells [30, 37]. Briefly, the separation channel (4.0 cm in breadth, 30 cm in length) was cut from a 0.025-cm thick Mylar spacer that was then placed between two transparent biocompatible plastic plates, namely between polycarbonate (PC) and polyvinyl chloride (PVC) walls, both from Plasticenter (Bologna, Italy). The flow of mobile phase (constituted by LB broth) was generated by a peristaltic pump (Miniplus 3, Gilson), while samples were injected by means of a 10-cm PEEK inlet tube employing a HPLC syringe. In particular, 150-μL aliquots of bacteria samples were manually shaken for few seconds, then injected at a flow rate of 0.2 mL min^{-1} for 15 s. Upon a 30-min stop flow to allow sample relaxation, the flow of mobile phase was restarted at 1.0 mL min^{-1} for samples elution.

In order to visualise the fractionation profile, a signal from the eluted cells was recorded at 600 nm by a UV 6000 LP fibre optics UV–visible (UV/vis) diode-array detector (ThermoQuest, Austin, TX) equipped with a 5-cm light-pipe cell. The UV/vis detector, which acts as a turbidimeter in this configuration providing a non-specific signal related to the presence of macromolecules (e.g. milk proteins) or cells in the eluting volume, was employed to monitor the fractionation profile of analysed samples during method optimization. Due to the high reproducibility of separation process, the UV/vis detector can be eliminated once the method was developed.

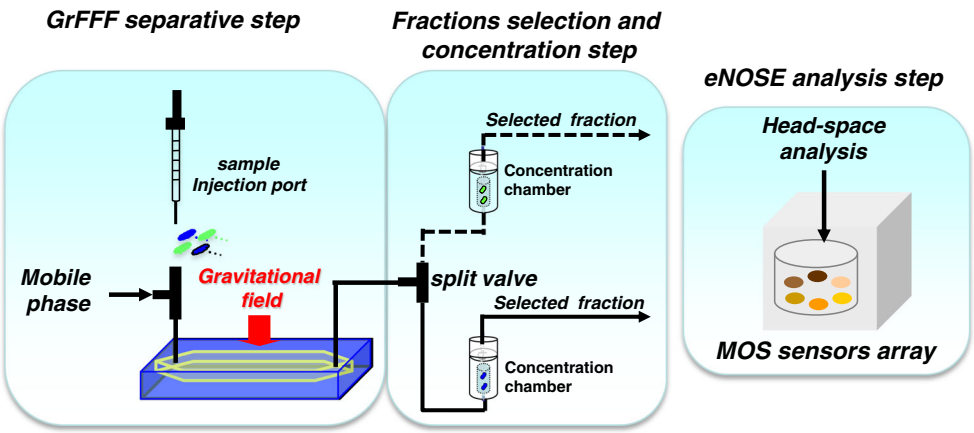


Fig. 1 Scheme of the GrFFF-MOS system: separative step; on-line fraction selection and concentration step; olfactory analysis step of selected fractions

At the channel outlet, an on-line split valve was placed in order to direct a selected fraction to an on-line concentration chamber consisting of a 1-mL tube equipped with a 0.45-μm filter used to collect and concentrate fractions selected from repeated runs in a volume suitable for the direct analysis with MOS array.

Olfactory sensor module

The selected fractions obtained with the GrFFF module were analysed with the electronic nose EOS 835 (Sacmi Imola s.c.a.r.l., Imola, Bologna, Italy) [38], comprising an array of six thin-film MOS (SA0216, SB0207, SD0325, SH0325, SJ0711, SU0314). Measurements were performed by dynamic headspace using an automated sampling system.

Each selected sample (1 mL) was recovered from the concentration chamber and directly placed in a 15-mL airtight glass tube and incubated at 37 °C for 15 min just before sampling the headspace. The tube was thermostated at 37 °C during analysis. Bacteria growth during the analysis was monitored by comparing the optical density at 600 nm measured at the beginning and at the end of the MOS array measurement.

The headspace gas mixture was transported to the sensors at a 150 standard cubic centimetres for minute (sccm) flow. The sensor chamber temperature was set at 55 °C, while the MOS sensor temperature was set at optimal values suggested by the manufacturer, all comprised between 350 and 450 °C.

For each fraction, a sequence of five cycles of exposure to the six sensors was performed, each consisting of four steps:

1. 'Before' (0.5 min, flushing reference air to measure the baseline signal);
2. 'During' (0.7 min, exposing the sensors to the sample, absorption phase);
3. 'After' (10 min, flushing reference air to allow sensors to return to their baseline value of resistance, desorption phase);
4. 'Wait' (13 min, flushing reference air before the following measurement cycle).

The resistance values recorded in the steps during and after were employed for signal processing.

At the end of each analysis, the tube system has been cleaned with ethanol and dried with nitrogen, in order to eliminate residual traces of the sample analysed. The GrFFF-MOS system can be reused for several analyses, up to 50 runs, without loss of sample recovery and separation efficiency, then plastic channel walls can be replaced.

Data analysis

For each measurement, the acquired data (raw data) were first subjected to feature extraction employing the 'many contiguous points' algorithm of Nose Pattern Editor software provided with EOS835, which calculates the value of resistivity for each sensor at 13 different characteristic points (hereafter called features) of its response curve, thereby obtaining 78 variables, whose values constitute the sample olfactory fingerprint. The procedure, previously employed [45] is described in the Supplementary material. Features obtained in five consecutive cycles of exposures comprised in one measurement session were averaged.

In this study, three different data sets were considered in order to demonstrate the ability to recognise viable cells from non-viable (1, 2). A third data set (3) was employed to investigate the feasibility towards a multiplexed analysis to detect and/or identify more than one pathogen in a single analytical run:

1. Viable/non-viable *E. coli*: this data set is composed of the fingerprints of 10 samples of viable *E. coli*, 15 samples of non-viable *E. coli*, 5 mixtures 1:1 viable/non-viable *E. coli* in LB broth (for a total of 10 fingerprints) and 5 mixtures 1:1 in skimmed milk (other 10 fingerprints); thus the final data matrix contains 45 rows and 78 columns (samples and variables, respectively).
2. Viable/non-viable *Y. enterocolitica*: this data matrix is formed as the previous one but with *Y. enterocolitica* fingerprints instead of *E. coli* ones.
3. Viable *E. coli* and viable *Y. enterocolitica*: this data set is composed of the fingerprints of ten samples of *E. coli*, ten samples of *Y. enterocolitica*, five mixtures 1:1 in LB broth (for a total of ten fingerprints) and five mixtures 1:1 in skimmed milk (other ten fingerprints); thus, the final data matrix contains 40 rows (samples) and 78 columns (variables, features).

The olfactory sensors array requires an accurate training set in order to be subsequently used for unknown samples recognition. In this work, for each data matrix, the fingerprints obtained by analysing GrFFF fractions collected from injections of bacteria (either single strains or binary mixtures) prepared in LB broth were used as a training set and the fingerprints obtained by GrFFF fractions collected upon injection of binary mixtures in skimmed milk were used as unknown samples (test set).

Data processing was performed by employing multivariate techniques with the V-PARVUS chemometric statistical analysis programme. In particular, after a preliminary data scaling (column autoscaling), PCA [39] was used as a display method, while LDA [40] was applied as a classification tool in order to discriminate between different bacteria strains and between viable/non-viable bacteria of a given strain. LDA is a probabilistic classification technique which searches for directions (canonical variables) with maximum separation among categories; the first canonical variable is the direction of maximum ratio between interclass and intraclass variances.

LDA was always performed on the first principal component (PC) scores, seeing as a reduction in size was necessary for all three data matrices, considering the ratio between the number of variables and the number of samples. The number of significant PCs was chosen on the basis of the prediction ability in cross-validation.

The LDA classification rules were validated by means of a cross-validation procedure with five cancellation groups (five CV) and their performances tested on the external test sets.

Results

MOS sensors analytical performance

First, single-component samples containing only one analyte of interest (i.e. viable *E. coli*, non-viable *E. coli*, viable *Y. enterocolitica* or non-viable *Y. enterocolitica*) were processed with the proposed analytical platform in order to analyse the instrumental output signals.

Viable *E. coli* and *Y. enterocolitica* (10 samples each) and non-viable *E. coli* and *Y. enterocolitica* (15 samples each) were analysed with the proposed GrFFF-MOS platform. Figure 2 reports the typical fractographic profiles. The retention time maximum for non-viable cells was different from that of viable cells, mainly because of changes in membrane permeability and rigidity which determine the loss of intracellular contents upon cells death [41].

Figure 3 (left) reports a typical olfactive fingerprint, based on the response of six MOS sensors, of a fraction of viable *E. coli* cells collected upon GrFFF fractionation. It can be observed that the six MOS sensors respond to a different extent to the VOC composition of the sample headspace, the sensor SH0702 providing a much stronger signal (2 to 4 orders of magnitude higher) with respect to the others. Nevertheless, more than basing on the intensity of each sensor signal to a given sample, the usefulness of each sensor in the MOS array in defining a distinctive olfactive fingerprint useful for discriminating and correctly classifying different cells populations must be evaluated basing on its ability to provide different response to different samples. Figure 3 (right) reports olfactive fingerprints obtained by subjecting to MOS array measurement a fraction of viable *E. coli* cells and a fraction of non-viable *E. coli* cells collected upon GrFFF fractionation of single cell populations. For each sensor, the signals have been normalised to their highest value in order to evidence differences between the olfactive fingerprints of the two samples. It can be seen that all the six MOS sensors provide information useful for samples discrimination, irrespectively to their ability to provide high or low signals when exposed to the headspace VOCs. Thus the combined analysis of the six MOS sensor responses through a chemometric approach is necessary to maximise sample classification ability. Same MOS results were obtained for *Y. enterocolitica* samples (data not shown). While not in the scope of this paper, it could be useful in a future work to explore by a chromatographic approach (e.g. GC-MS analysis) the different VOC compositions of the headspace sample obtained from viable or non-viable bacteria of the same strain.

GrFFF-MOS analysis of viable and non-viable cells of the same strain

Viable and non-viable E. coli

Mixtures of 1:1 viable and non-viable *E. coli* bacterial cells in LB broth (five samples) were injected in the GrFFF module.

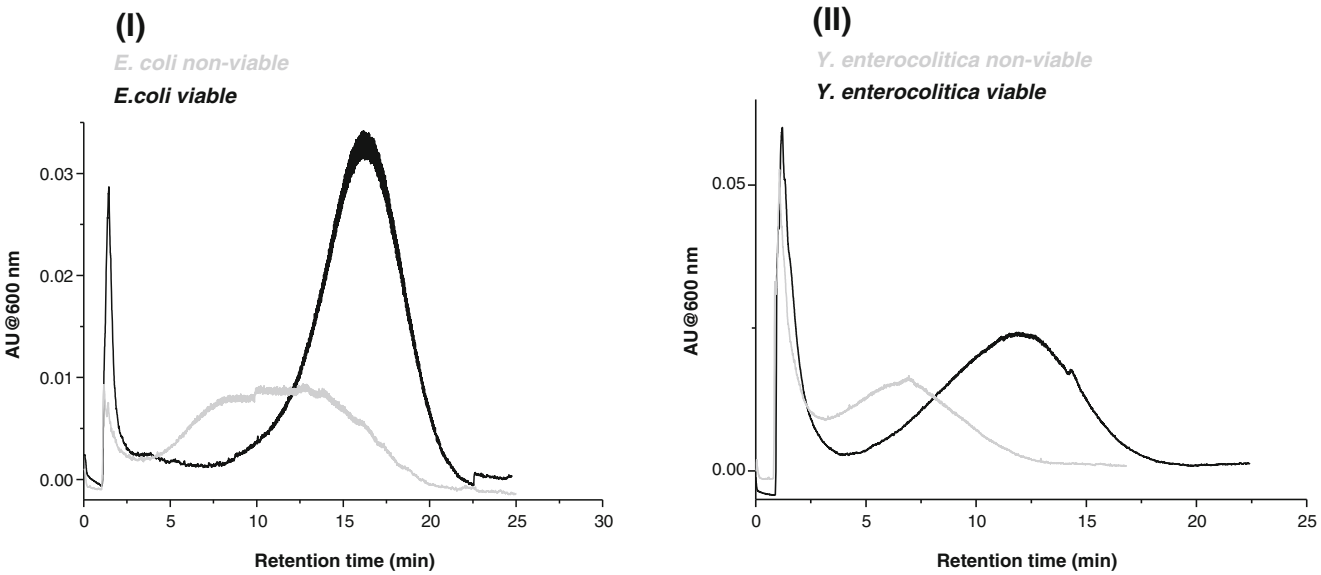
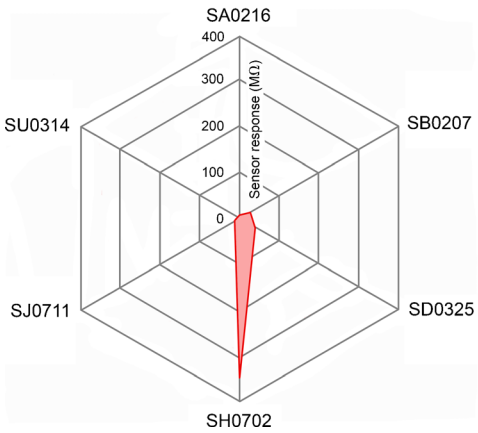


Fig. 2 Fractograms obtained upon separate injection of viable and non-viable *E. coli* (*I*) and viable and non-viable *Y. enterocolitica* (*II*)



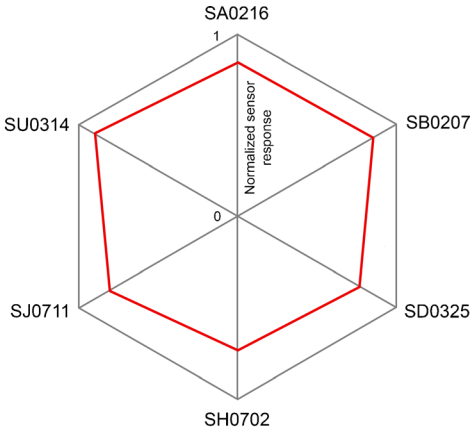


Fig. 3 *Left*, typical olfactive fingerprint, based on the response of six MOS sensors (calculated as the average of resistance in Ohm measured when the sensor was exposed to the sample), of a fraction of viable *E. coli* cells collected upon GrFFF fractionation. *Right*, olfactive fingerprints obtained by subjecting to MOS array measurement a fraction of viable *E. coli* cells collected upon GrFFF fractionation and a fraction of non-viable *E. coli* cells subjected to the same procedure. For each sensor, the signals have been normalised to the signal obtained for non-viable *E. coli* in order to evidence differences between the olfactive fingerprints of the two samples, irrespectively to the absolute value of sensor signals

The GrFFF profile (Fig. 4) obtained for the mixture in LB broth shows two main bands at retention times typical for non-viable and viable cells. Five skimmed milk samples contaminated with a 1:1 binary mixture of viable and non-viable cells were also analysed. The typical GrFFF fractographic profile (Fig. 4) shows a first broad main band (eluting before 5 min) due to the high content of small unretained species in milk (submicron particles such as proteins, lipids) covering the retention interval of non-viable cells and a second band at retention time typical for viable *E. coli* cells. As the fractogram acquired with UV/vis detector does not provide conclusive information on the cell populations present in the sample, due to the non-specificity of the detector, subsequent MOS array analysis was performed on collected fractions to classify bacterial cells present in the sample. Fractions corresponding to elution time characteristic of viable (F2; 17–20 min) and non-viable (F4; 7–10 min) *E. coli* already determined by injections of single cell population were selected and subjected to MOS array analysis.

Figure 5 shows the scores on the first two principal components. It is possible to notice that the first principal component, which is the direction of maximum explained variance (85.5 %), demonstrates a perfect separation between viable and non-viable *E. coli*; moreover, all the test set samples (F2 and F4 fractions from mixtures in milk) were correctly positioned within the corresponding group. Then, LDA (Table 1) was applied as a supervised method, in order to classify the bacteria according to their strain. Seeing the number of high variables if compared with the number of samples, LDA was always applied working on the scores of the first principal components. For each, data set, the two first significant principal components were retained, in this case corresponding to

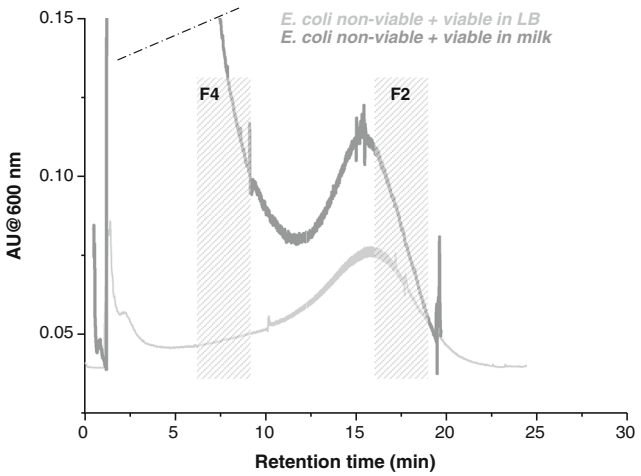


Fig. 4 Fractograms obtained upon injection of a binary mixture containing approximately the same number of viable and non-viable cells in LB broth or in skimmed milk. Collected fractions: *F4* non-viable *E. coli* (7–10 min), *F2* viable *E. coli* (17–20 min)

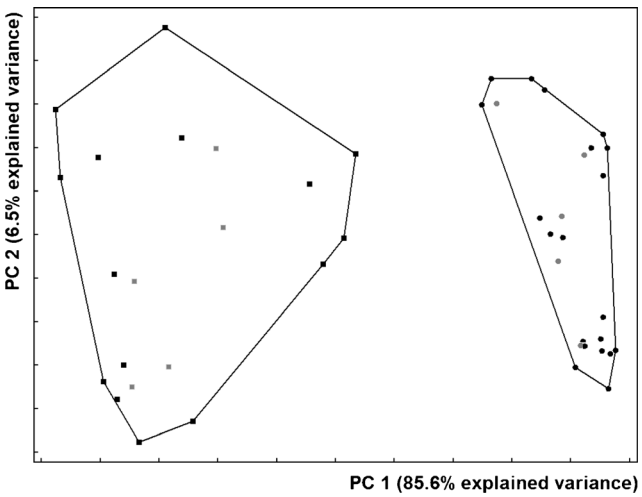


Fig. 5 Score plot on PC1–PC2 obtained by the viable/non-viable *E. coli* data set. Samples are represented by a class symbol (*squares* viable *E. coli*; *circles* non-viable *E. coli*). Test set samples in *grey*

about 90 % of the total variance. In Table 1, the LDA results are reported as correct prediction rate in cross-validation (five CV groups) and correct prediction rate on the external test set. Excellent results were obtained achieving a mean of 100.0 % prediction ability both in cross-validation and on the external test set.

Viable and non-viable Y. enterocolitica

To demonstrate the general applicability of the proposed approach to other pathogen bacterial strains, the same experiments were performed by injecting in the GrFFF system mixtures of 1:1 viable and non-viable *Y. enterocolitica* in LB broth (five samples) or in skimmed milk (five samples). Fractograms are reported in Fig. 6. For each run, F1 (viable, 10–13 min) and F3 (non-viable, 5–8 min) fractions were collected. Results obtained upon MOS array analysis and chemometric approach (Table 1) demonstrate similar performance as those obtained for viable and non-viable *E. coli*.

Measurements of OD values at 600 nm performed at the beginning and at the end of the 2-h window for MOS array measurement confirmed that no bacterial growth was observed in fractions F3 and F4, in which non-viable cells were collected.

GrFFF-MOS analysis on *E. coli* and *Y. enterocolitica* cells

Viable *E. coli* (ten samples), *Y. enterocolitica* (ten samples) and 1:1 (approximately equal number of cells) mixtures of the two bacterial strains (five samples) in LB broth were fractionated by GrFFF and visualised using an UV/vis detector at 600 nm. Typical fractographic profiles (Fig. 7, I) show that the two strains have different elution profiles, due to their different morphological properties. In accordance with the FFF theory, *Y. enterocolitica* (rod shaped, 0.4–7 μm length) elute before *E. coli* (curved rod shaped, 0.6–5 μm length). Upon injection of a 1:1 mixture of the two species, yielding a broad band, fractions corresponding to elution time characteristic for each strain were collected: F1 (from 10 to 13 min) for *Y. enterocolitica* and F2 (17–20 min) for *E. coli* (Fig. 7, I).

Each collected fraction was subjected to MOS array analysis (15 samples for each fraction, F1 and F2) and results were used as a training set for chemometric analysis.

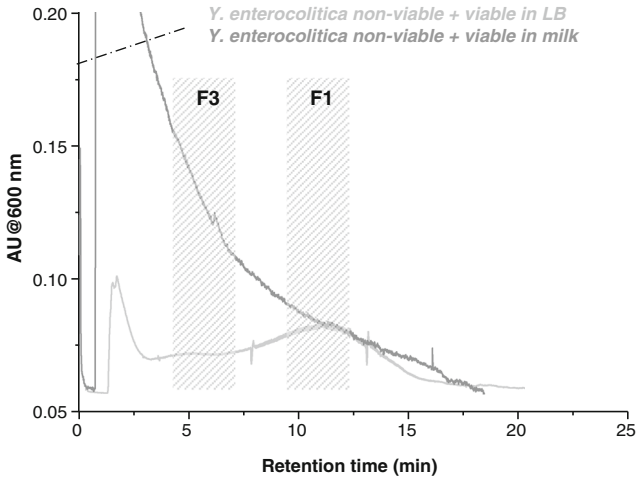


Fig. 6 Fractograms obtained upon injection of a binary mixture containing approximately the same number of viable and non-viable cells in LB broth or in skimmed milk. Collected fractions: *F3* non-viable *Y. enterocolitica* (5–8 min), *F1* viable *Y. enterocolitica* (10–13 min)

Five skimmed milk samples contaminated with a 1:1 binary mixture of the two strains were also analysed (Fig. 7, II). F1 typical for viable *Y. enterocolitica* cells and F2 typical for viable *E. coli* cells were collected: the ten obtained MOS array signals were used as a test set.

Figure 8a shows the scores on PC1–PC2 (78.0 % of the total explained variance): in this plot, the two bacterial strains were distributed in two clusters completely separated along PC2. As far as the test set is concerned, all samples were correctly positioned within the corresponding cluster.

Figure 8b shows the histogram on the LDA canonical variable: squares represent *E. coli* class, while triangles represent *Y. enterocolitica* samples. As expected from the PCA result, the data present a 'bimodal distribution' on the canonical variable, with two separated groupings corresponding to the two bacteria strains.

To stress the complexity of the separation step, the same experiments were also performed on mixtures of the two bacterial strains (in LB broth or in skimmed milk) prepared with a 4-fold excess of one strain with respect to the other: 20 % *E. coli* and 80 % *Y. enterocolitica* cells or 80 % *E. coli* and 20 % *Y. enterocolitica* cells and fractions F1 and F2 were collected and analysed by MOS array, obtaining similar results.

Table 1 LDA results reported as correct prediction rate in cross-validation (five CV groups) and correct prediction rate on the external test set

Data set	Variance explained by the 2 first PCs (%)	Prediction rate (5 CV; %)	Prediction rate (test set; %)
Viable/non-viable *E. coli*	92.0	100	100
Viable/non-viable *Y. enterocolitica*	95.7	100	100
Viable *E. coli* and *Y. enterocolitica*	78.0	100	100

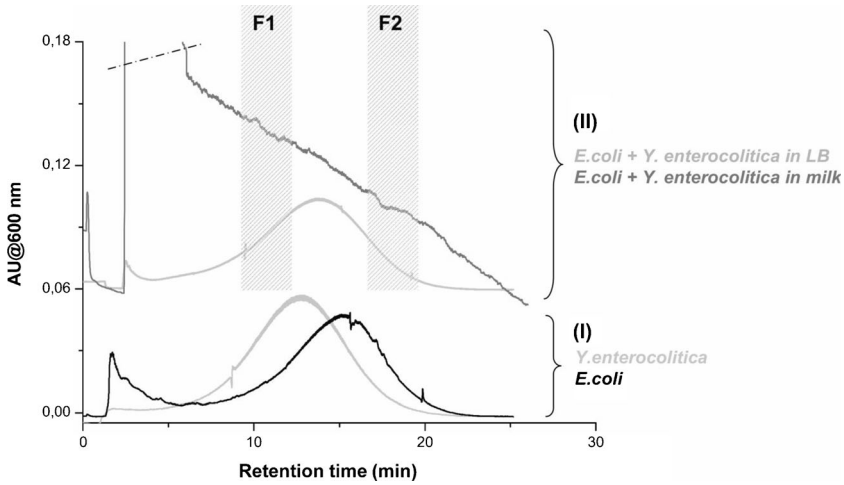


Fig. 7 Fractograms obtained upon separate injection of viable *E. coli* and viable *Y. enterocolitica* (*I*) or injection of a binary mixture of the two strains containing approximately the same number of cells for each strain in LB broth or in skimmed milk (*II*). Collected fractions: *F1 Y. enterocolitica* (10–13 min), *F2 E. coli* (17–20 min)

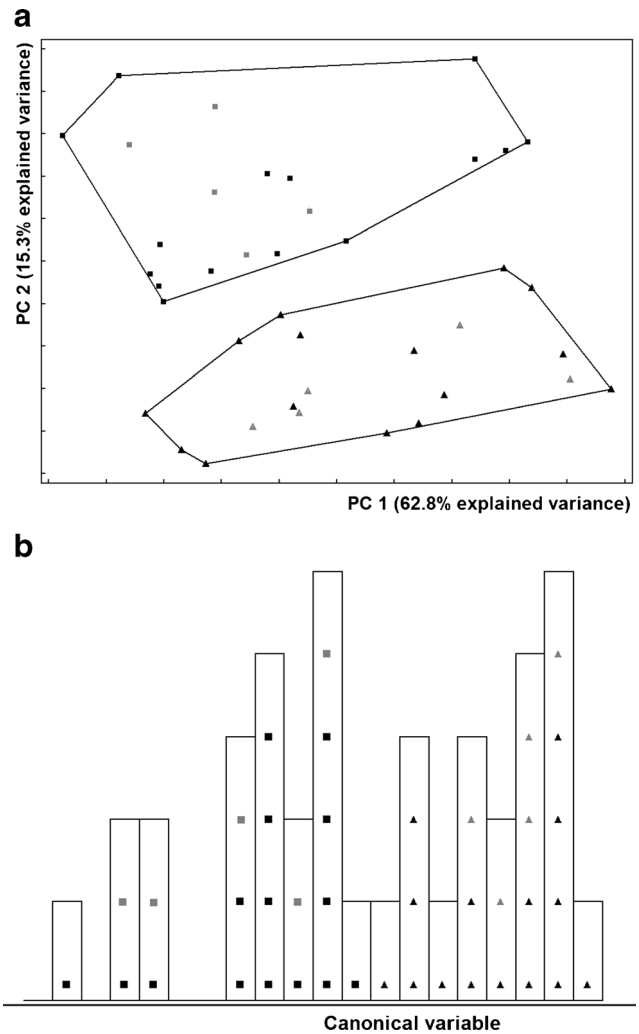


Fig. 8 **a** Score plot on PC1–PC2 obtained by the *E. coli* and *Y. enterocolitica* data set. Samples are represented by a class symbol (*squares E. coli*, *triangles Y. enterocolitica*). Test set samples (*E. coli* and *Y. enterocolitica* binary mixtures in skimmed milk) in *grey*. **b** Histogram of LDA scores on the canonical variable obtained for the analysis of *E. coli* (*squares*) and *Y. enterocolitica* (*triangles*) cells by GrFFF-MOS system. Test set samples (*E. coli* and *Y. enterocolitica* binary mixtures in skimmed milk) in *grey*

Discussion

The main aim of the paper is to demonstrate that GrFFF fractionation is able to prepare, from a complex sample, a fraction enriched in the bacterial population of interest (e.g. viable cells) and devoid of matrix interferents, thus significantly facilitating and improving the identification ability of MOS array analysis.

The ability of the GrFFF device to fractionate viable and non-viable cells also in real matrices was demonstrated through the analysis of samples containing mixture of two bacteria. The GrFFF module was able to separate bacteria from mixtures, maintaining typical retention times for each species and providing band intensity proportional to sample quantity. MOS array analysis was able to identify non-viable bacteria of a given strain exploiting its distinct olfactive fingerprint. As stated above, the ability to identify non-viable bacterial cells of a pathogenic strain in a food sample is one of the main challenges in food safety assessment. This is because, even in the absence of viable bacteria that would directly exhibit their pathogenic potential, a past contamination with toxigenic bacteria might have caused contamination with toxins that are usually heat resistant. Although our integrated GrFFF-MOS sensing system is not proposed for direct toxin determination, its ability to detect non-viable bacterial cells of a given strain upon proper training can be exploited as a simple and rapid screening test, prompting further analysis to assess toxin contamination only of those samples where the corresponding bacterial cells have been detected.

Bacteria were killed by exposure to heat. We expect heat treatment to induce expression of heat shock proteins and, upon cell membrane damages, release of heat-stable proteases and lipases. Such enzymes could cause breakdown of proteins and lipids present in the LB broth and, together with possible degradation of cellular components, determine a specific VOC composition of the sample head space during the MOS

array analysis. Preliminary experiments showed that the olfactory fingerprint of heat-killed *E. coli* was clearly differentiated by LDA analysis from that of heat-killed *Y. enterocolitica* (ability of correct classification and prediction 100), indicating that VOC composition is species specific even when dead cells are analysed. To the best of our knowledge, this is the first time that such result is shown for electronic nose analysis.

Another important feature of preliminary GrFFF analysis is the ability to reduce sample complexity, eliminating cell debris, proteins and matrix components. Milk is a relatively complex matrix, which contains significant amounts of lipids, carbohydrates and proteins that influence the bacteria metabolism. In addition, different milk samples might present differences in their composition, thus causing variability in the VOC composition of the headspace due to metabolic changes induced in cells. With this respect, the preliminary GrFFF step that eliminates milk components and provides an enriched bacteria strain in mobile phase (LB broth in this work) is crucial to increase reproducibility of the analysis and to allow direct MOS array analysis.

In addition, we demonstrated that, thanks to the coupling to GrFFF, a MOS array sensor training performed employing bacteria in LB broth could be used to correctly identify bacteria added to a complex food matrix. This greatly simplifies procedures for pathogenic bacteria identification in food samples, with consequent reduction in the assays cost and time, since one common training in LB broth can be used for analyzing different types of milk (e.g. skimmed, semiskimmed, whole), without the need for a specific training for each matrix. In this context, it will also be necessary to evaluate whether bacteria of the same strain grown in different food matrices, or in a given food matrix but in different conditions, might display different behaviour during GrFFF fractionation. In the future, this aspect, together with the ability of the GrFFF-MOS system to act on different types of food matrices (e.g. different milk types or fruit juices) will be also explored, as well as the influence of food conservation conditions on the assay reproducibility.

Detectability offered by the GrFFF-MOS hyphenated method was also evaluated, considering that a volume of 150 μL of sample containing 2.4×10^9 CFU mL^{-1} bacterial cells was injected in the GrFFF module, followed by fractions collection and MOS array analysis, and that a nearly total cell recovery was previously demonstrated [42]. As the developed fractionation method results highly reproducible, to increase detectability in real samples, it is reasonable in a daily procedure to pool in the 1-mL-volume collection tube fractions collected from eight consecutive runs (total time for fractions collection 2 h using two GrFFF parallel systems), thus enabling target bacteria detection in milk samples contaminated with 3.6×10^8 CFU mL^{-1}. This detectability is comparable with, or in some cases better than, those previously obtained for bacteria species discrimination employing other olfactory systems [3, 14, 43]. It has to be underlined that these results refer for the first time to the direct analysis of complex matrices, thus the obtained sensitivity, although still improvable by instrumental and methodological modifications, can be considered acceptable since it provides a robust, reproducible and fast method for the classification and identification of different bacteria directly in their food matrix. The GrFFF-MOS method was developed and characterised using a 1:1 mixture of viable and non-viable cells. In addition, preliminary data indicate that similar results can be obtained with different proportions of viable and non-viable cells (30/70 and 70/30) of the same strain. A method validation can be performed in order to determine the selectivity of this approach.

In this work, a preliminary result on the use of GrFFF-MOS system for the analysis of different bacteria strains in mixture was also presented.

Although GrFFF yields fractions enriched in the target bacterial population, rather than total separation, collected bacteria could be successively identified by MOS array analysis and chemometric data processing. This was demonstrated even in unfavourable situations, such as mixtures in which one bacterial strain is in large excess with respect to the other. Although deeper investigation is required, this latter experiment provides a preliminary indication that a given bacterial cell population can be correctly classified upon GrFFF slicing, MOS array analysis and chemometric approach, even if present in the sample at a concentration different than that employed during the training procedure. Thus, GrFFF band slicing can adequately enrich each fraction on the target bacterial strain for subsequent MOS array-based identification.

Each fraction was analysed five consecutive times with the MOS array during exponential bacterial growth (as evidenced by measuring the optical density at 600 nm at the beginning and at the end of the measurement), thus maximising the ability of MOS array analysis to distinguish between different bacterial strains on the basis of the dynamics of VOC production due to their active metabolism.

In the future, the use of a 2D FFF separation strategy, in which each fraction collected from a first GrFFF channel is further fractionated by a second FFF channel, could be explored to increase fractionation efficiency prior to MOS array analysis, thus allowing identification of other pathogen bacteria potentially present in a milk sample. Reinjection of one or more collected fractions of eluted samples is recognised as a useful procedure in analytical separation techniques, among which FFF, to improve the actual separation of complex samples [44].

Conclusions

Obtained results show that the combination of GrFFF separation, MOS array analysis and multivariate data processing (chemometrics) can significantly increase reliability and

reproducibility of MOS array identification, simplifying the analytical procedure and largely eliminating sample matrix effects. The developed GrFFF-MOS system can be considered a simple straightforward approach for pathogen bacteria identification directly from their food matrix. Viable and non-viable cells could be clearly distinguished within each bacterial species. The system could be employed in quality control assays included in procedures for food analysis and safety control, providing the ability to evaluate bacteria viability in foodstuff in a relatively short time, offering also the opportunity to separate and analyse cells.

In the future, the overall assay time can be significantly reduced and sensitivity improved by enhancing the discrimination ability of the MOS array analysis, for example, by increasing the number of sensors in the array, by introducing sensors calibration procedures and humidity control systems that would reduce response variability or by selecting a different culture media as the GrFFF mobile phase in order to increase dynamic metabolic differences among strains.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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