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A new efficient sample pooling procedure for qualitative screening analysis. Application to the detection of *salmonella spp* and nut allergen by PCR

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ABSTRACT

Foodborne pathogens and allergens are a major concern for public health that determines food safety policy, and their screening could be improved using pooled samples.

The purpose of this paper is to propose a cost-effective and accurate pooling strategy for the unambiguous identification of foodborne pathogens and allergens, enabling laboratories to redouble testing capacity while saving time and making optimal use of resources.

Although a variety of pooling algorithms have been used in different fields, the strategy suggested here is a logical analysis of sample pooling aimed at qualitative analytical screening problems. It involves, on the one hand, a design matrix to make the pooled samples, which is a supersaturated-based design, particularly, a half-fraction of a Plackett-Burman. On the other hand, a logical (non-algebraic) modeling of the problem, as well as a logical procedure for the identification of the original positive samples, is included.

Regarding the efficiency of the proposal, it is higher than that of other pooling algorithms, with an expected number of tests per individual sample ranging between 0.10, for a prevalence below 1%, and 0.59, for a prevalence above 10%. In terms of accuracy, the pooling sensitivity reaches 0.9697 for a sensitivity of the analytical test about 0.99, while pooling specificity ranges from 0.9872 to 0.9999, provided that the sensitivity and specificity of the analytical test are equal to or greater than 0.90.

This pooling strategy has been applied to the detection of *salmonella spp* and nut allergen with complete identification of the contaminated samples. Furthermore, based on the EU reported food samples contaminated with *salmonella*, a comprehensive comparison between individual sampling and the applied pooling strategy has been conducted. For the specific case of *salmonella*, detailed calculations have been made on the expected efficiency gains induced by this pooling methodology for different types of food samples monitored in the EU.

1. Introduction

Food safety is critical to achieve the Sustainable Development Goals (SGD). Feeding a growing world population through a fair, healthy, and environmentally friendly food system, in a context of unstable supply driven by climate change, requires regulatory harmonization and strong data-based risk management [1,2].

As food supply involves supply chains across multiple countries, the Food and Agricultural Organization of the United Nations (FAO) and World Health Organization (WHO) provided a set of international standards (Codex Alimentarius) to promote fair practices in food trade and protect consumer health. The current food safety strategy proposed by the WHO is based on the 'One Health' approach, which aims to optimize the health of humans, animals and ecosystems [3]. The Global Food Safety Initiative covers the entire food chain, 'Farm to Fork', underpinning the safety of the global food chain and fostering regulatory compliance, trade, and consumer trust [4].

According to WHO, almost 1 in 10 people fall ill eating contaminated food worldwide, straining health care systems and damaging national economies, tourism, and trade, which results in an estimated global annual burden of 33 million disability-adjusted life years (DALYs) [3,5].

Foodborne illnesses are caused by food safety hazards, whether biological (such as bacteria or viruses), chemical (for example, heavy metals or pesticides) or physical. Many foodborne diseases are zoonotic, transmitted from animals to humans, and others can be caused by multiple factors, such as food allergies and intolerances.

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Regarding zoonoses, the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) supply detailed information on zoonotic microorganisms in food and feed sources, food-producing animals, companion and wild animals, and foodborne disease outbreaks in the EU and other European countries. In 2022, *Campylobacter* and *Salmonella* have caused the two most reported zoonoses in humans, surpassing 200,000 cases [6], and *Salmonella* is the most frequent cause of foodborne outbreaks in Europe. In the US, the Centers for Disease Control and Prevention (CDC) monitor bacterial foodborne illnesses triggered by *Salmonella*, *Campylobacter*, *Listeria* and up to nine pathogens, estimating the costs in terms of loss of productivity and medical expenses. *Salmonella* has been the leading cause of Intestinal Disease Outbreaks in 2017–2020 in the US [7] and its reduction is one of the goals to improve health in the coming years [8].

As to food allergens, EFSA defines allergenicity as the "ability to trigger an abnormal immune response that causes an allergic reaction in a person''. A limited number of proteins that make up the human diet are potential inducers of food allergies. In developed countries, their prevalence ranges from 6 to 8% in children and 2–3% in adults and is growing in the past 20 years [9]. An updated systematic review and *meta*-analysis in Europe [10] provides a point prevalence of self-reported Food Allergy of 13.1% (95% CI 11.3–14.8).

According to the Food and Drug Administration (FDA), ingredients related to nine foods (milk, eggs, fish, crustacean shellfish, nuts, peanuts, wheat, soybeans, and sesame) account for more than 90% of allergic reactions, a major public health problem in the US, so they must be disclosed. Similarly, in the EU, fourteen allergenic foods or any product derived from these foods, must be labelled, following the regulation on the provision of food information to consumers [11]. More than sixty studies in 41 countries on the proportions of anaphylaxis due to specific food triggers have been gathered in [12].

Due to their high nutritional quality, nuts are considered a part of a healthy diet so an increasing consumption (snacks, part of different food products, ingredients in a range of dishes) and exposure (through cosmetics, pet food handling, or food processing) occurs. Furthermore, the high costs of nut production make them susceptible to fraud [13] which affects the allergenicity and risk to consumers.

A summary of the knowledge on nut allergens -physicochemical and immunological characterization and clinical relevance- can be found in [14], where a prevalence of nut allergy of 4.9% worldwide is reported. More specifically, FAO and WHO [15,16] report on proportions of individuals allergic to cashew, hazelnut and walnut expected to have symptoms at different doses of exposure.

A review and validation of the Codex allergen priority list has recently been carried out [15,16] Regarding nuts, high prevalence is reported in Europe for cashew nut, pistachio, and hazelnut.

As to nut allergens, [17] provides an updated outline of the applications of real-time PCR for their detection in processed food products. To avoid a great health risk to sensitized individuals and massive costs to producers, analytical methods are required to reliably detect and identify allergens in food products.

According to EFSA Strategy 2027 [18], one of the main principles of food safety policy is that it should be evidence-based. Therefore, food chain data and analytics will reinforce forecasting and early warning systems, being crucial for food safety risk management.

In this context, screening methods for the unambiguous identification of harmful substances in food may benefit from sample pooling. When the number of samples with a trait of interest, 'positive' samples, in the entire population (prevalence rate) is low, testing individual samples involves a waste of available resources, since most of them will be negative. A pooled sample is a combination of small portions of individual samples, and the advantage of 'pooled testing' is to discard individual samples which cannot be 'positive', thus meaning a reduction in the total number of analytical tests performed. For a low prevalence and suitable group sizes, pooling strategies can mean a substantial decrease in the expected number of tests compared to individual tests.

To increase laboratory testing capacity, sample pooling has been applied to the detection of infectious diseases (sexually transmitted diseases, HIV, Hepatitis, COVID-19 [19,20,21]) and to other areas of biomedicine (blood donation tests, drug screening), as well as to environmental studies or veterinary research.

For the purpose of this paper, it is imperative to summarize its application to salmonella detection in eggs [22], poultry [23], pigs [24,25], bovine [26], horses [27] or different foods [28].

Generally, analyses of sample pooling outcomes have been primarily conducted with regression models (see ref. [29] and papers cited therein for further details). However, a different approach to these analyses has recently been suggested in ref. [30]. The central idea is to consider that the outcome of a qualitative assay such as PCR is binary: either the pooled sample is negative or positive. However, the latter occurs if there is at least one positive original sample, so a pooled sample is 'simply positive' regardless of the number of positive samples included in it. Therefore, the outcome of the pooled samples is not governed by the algebraic operation 'sum' of the individual results, but by the logical operator 'V' ('or' in natural language).

The methodology developed in [30] for obtaining the pooled samples and the identification of the true positive samples has been applied for the first time to the detection of the pathogen *Salmonella spp* and to the DNA of nuts, by polymerase chain reaction (PCR).

In section 2, after an outline of sample pooling algorithms (section 2.1), the suggested pooling strategy is detailed in sections 2.2, pooling design matrix, and section 2.3, logical formalization of sample pooling and determination of the original samples to be analyzed individually, including some scenarios to illustrate the rationale of the procedure. The assessment of the proposal is based on the usual figures of merit, namely, efficiency, accuracy, and pooling sensitivity and specificity. Comparisons with the most common pool testing algorithms are presented in section 2.4. The software and the experimental are explained in section 3. The detection of food pathogens and allergens through this procedure is showed in section 4.1, for both *Salmonella* and *nut* allergens. Additionally, a detailed study on efficiency of SS-based pooling for salmonella is included in section 4.2. The paper ends with some conclusions and references.

2. Proposal

The use of pooled samples to identify the individual (original) 'positive' samples may be referred to as 'pooled testing', also known as 'group testing' or even 'sample pooling' or 'specimen pooling'. If a 'pooled sample' tests negative, none of the original samples included in it needs to be re-tested, so all of them are discarded. When the pooled sample tests positive, at least one of its original samples is positive so further investigation is needed, either individual analysis of each original sample or additional sample pooling steps to achieve unambiguous identification.

An extensive survey of recent developments on pooled testing and the underlying mathematical theory are provided in [31,32], including a classification of algorithms based on different criteria. According to the purpose of the analysis, sample pooling algorithms can be used to: i) estimate the prevalence of an effect (e.g. a disease) in a population, ii) identify individuals showing the effect (e.g. testing positive for the disease).

Many pooling algorithms are divided into hierarchical and non-hierarchical. The former test samples over multiple stages in non-overlapping pools, following the pioneer work of Dorfman [33].

The non-hierarchical algorithms test samples in overlapping groups at the initial stage, so an individual sample can be represented in more than a pool, with the aim of reducing the number of individual samples which have to go to a further stage, i.e., the number of retests. Array testing is a common non-hierarchical pooling algorithm. In 2D-array testing, the original samples are arranged in a square matrix $n \times n$, and $2n$ pooled samples are formed by combining the samples of each row

and each column [34].

A pooling procedure, developed in stages but not strictly hierarchical, fully detailed in [30] will be used in this work. To facilitate the reading of this paper, it is summarized in this section, including: i) the procedure for obtaining the pooled samples, ii) the logical modeling of the problem, and iii) the logical procedure that leads to the identification of the original positive samples.

2.1. Pooling design

Supersaturated (SS) designs of experiments have been used in chemometrics [35] to obtain the pooled samples in the analytical laboratory. In the context of sample pooling, the factors, columns in the SS design matrix, are the original samples from which the pooling is made, whereas the experimental units, rows in the SS design matrix, are the pooled samples. As each original sample may be included or not in a particular pooled sample, it is a 2-level design.

From original samples, S_i ,$i = 1,2,\cdots,I$, pooled samples P_t, $t = 1, 2, \cdots, T$ are generated using a design matrix $\mathbf{X} = (x_{ti})$, where $x_{ti} = 1$ if $S_i \in P_t$ and $x_{ti} = 0$ if $S_i \notin P_t$. The **X** proposed here is a half-fraction of a Plackett-Burman design with N rows and f columns PB(N,f). Particularly, a half-fraction of a PB(12,11) design is obtained using a branching column as in [36], so we are dealing with $f - 1 = 11–1$ factors, the $I = 10$ original samples, and a total of $N/2 = 12/2$ experiments, the $T = 6$ pooled samples to be analyzed.

The traditional notation of the SS design matrix (-1/+1) has been adapted to the usual notation of pooled testing (1/0). Table S1 in the supplementary material shows the design matrix, $\mathbf{X}_{6\times10}$, whose elements$x_{ti} = 1$ indicate the original samples S_i, $i = 1$, …, 10 (columns) combined in each pooled sample P_t, $t = 1$, …, 6 (rows).

2.2. Logical modeling of the problem

According to the outcomes of the analytical test, PCR, logical values are assigned to samples: the logical value is 0, if the statement 'the sample is positive' is false, and 1, if that statement is true. Both the I original samples, S_i, and the T pooled samples, P_t, are given their respective logical values: $lv(S_i)$ and $lv(P_t)$.

Since only pooled samples are analyzed by PCR, $lv(P_t)$ are known experimentally, while $lv(S_i)$ are the unknowns to be found. Using the design matrix $\boldsymbol{X} = (x_{ti})$, and the Boolean inclusive OR operator, $\vee$, a system of T logical equations is built, Eq. (1), with one equation for each P_t which reflects its composition:

$$lv(P_t) = \bigvee_{i=1}^{I} x_{ti} lv(S_i), t = 1, 2, \cdots, T \quad (1)$$

Being $y_t \in \{0, 1\}$ the outcome of the analytical test on the t-th pooled sample, y_t is also the experimental estimate of $lv(P_t)$. Therefore, the system of Eq. (1) becomes $y_t = \bigvee_{i=1}^{I} x_{ti} lv(S_i), t = 1, 2, \cdots, T$, where both the coefficients x_{ti} of design matrix **X**, and the outcomes of the pooled samples, y_t, are known, while the logical values identifying positive original samples, $lv(S_i)$, must be determined.

In short, the problem is formulated in such a way that it requires a logical solution, preventing the use of numerical methods to solve systems of algebraic equations.

2.3. Logical procedure for the identification of positive samples

The unambiguous identification of the positive original samples is carried out through the following three-step logical procedure:

Step 1: Analysis of the 'master pool', which appears as pooled sample number 6 in Table S1 (in the supplementary material). If the outcome is negative, then all the original samples are negative and the procedure ends. Otherwise, there is at least one positive sample and additional pooled samples are analyzed.

Step 2: Analysis of the remaining T-1 pooled samples, that is, P_1 to P_5 in Table S1 (in the supplementary material).

Step 3: According to the outcomes of the previous steps, it is logically decided which original samples cannot be positive. The remaining original samples are potentially 'positive' and are individually tested to confirm the truly 'positive' ones.

For illustrative purposes, three different scenarios have been simulated and included in Table 1.

In scenario 1, it is supposed that only two individual samples are contaminated, specifically S_1 and S_7. From the design matrix on Table S1 (in the supplementary material), we can obtain the logical value corresponding to the outcome of each pooled sample. In the first pooled sample, P_1, individual samples S_3, S_7, S_8 and S_{10} are combined, and as S_7 is contaminated, the outcome of P_1 will be positive, so the logical value is 1. In the second pooled sample, P_2, given that S_1 is contaminated, a combination of S_1, S_2, S_4 and S_{10} will also result in a positive outcome (logical value of 1). The rest of the logical values, pooled samples P_3 to P_6, are obtained with similar reasoning. Therefore, the logical process leads to discard only four samples, S_3, S_4 S_5 and S_9 (with '1′ in blue below in Table 1), keeping six individual samples as potentially positives (marked in red in Table 1). Note that only two of them are true positives, which will be identified in individual tests.

As to scenario 2, the supposed contaminated samples are S_8 and S_{10}. According to the design matrix, the first pooled sample, P_1, a combination of S_3, S_7, S_8 and S_{10}, will give a positive outcome (logical value, 1), while the third pooled sample, S_3, S_4, S_5 and S_9 combined, will be negative, corresponding a logical value of 0. After having logically discarded seven samples (with '1′ in blue below in Table 1), just three original samples (in red in Table 1) are potentially positives, although individual tests will detect the two true positives.

Finally, in scenario 3 only one contaminated sample is supposed, S_5. As the analysis of the six pooled samples leads to discarding nine samples, only S_5 (in red in Table 1) is candidate for retest and is finally identified as positive.

2.4. Figures of merit

2.4.1. Efficiency

Assuming perfect accuracy (perfect sensitivity and specificity), the evaluation of the pooling strategy suggested can be conducted in terms of efficiency, specifically, by the expected number of tests per individual (original) sample required to identify all the positives, E(T)/I. The computation of the expected efficiency has been detailed in Table S2 (in the supplementary material). The expected number of tests is the sum of the products of the probabilities of having i positive samples among the 10 used, which depends on the prevalence p, by the number of samples with i positives NT(i), Eq. (2):

$$E(T) = \sum_{i=0}^{i=10} p^i (1-p)^{10-i} NT(i) \quad (2)$$

Using Eq. (2) and a prevalence $p = 0.05$, the proposed algorithm has an efficiency, E(T)/I, of 0.37, so it is expected to perform 0.37 analytical tests on average to decide whether an original sample is positive or not, which means a reduction of 63% compared to individual tests.

A comprehensive comparison of the efficiency, in terms of E(T)/I, of the pooling strategy described with those of alternative pooling algorithms can be found in ref. [30].

Fig. 1, E(T)/I versus prevalence p, shows that the lower p, the greater the efficiency of any pooling procedure. With the suggested algorithm (red line), a prevalence of 0.10 results in an efficiency of 0.59, but when the prevalence drops to 0.01, efficiency is 0.16, representing a reduction in the expected number of tests per individual sample of 41% versus 84%. Naturally, for extremely low values of prevalence, p less than 10^{-3}, the efficiency stabilizes around 0.10.

This pattern of efficiency versus prevalence is very similar to that of the optimized hierarchical algorithms, specially 3-S (grey line), whereas the efficiency of 2D-array pooling algorithms, with or without master

Table 1
Design matrix for pooling (columns 2 to 11), '1' if $S_i \in P_j$, '0' if $S_i \notin P_j$. Logical values of the pooled samples according to PCR outcome (column 12):

Pooled samples	Original samples										Logical value
	S_1	**S_2**	**S_3**	**S_4**	**S_5**	**S_6**	**S_7**	**S_8**	**S_9**	**S_{10}**	
P_1	0	0	1	0	0	0	1	1	0	1	1
P_2	1	1	0	1	0	0	0	0	0	1	1
P_3	0	0	1	1	1	0	0	0	1	0	0
P_4	1	0	0	0	1	1	0	1	0	0	1
P_5	0	1	0	0	0	1	1	0	1	0	1
P_6	1	1	1	1	1	1	1	1	1	1	1

Scenario 2. The pooling results in three potential positive samples

Pooled samples	Original samples										Logical value
	S_1	**S_2**	**S_3**	**S_4**	**S_5**	**S_6**	**S_7**	**S_8**	**S_9**	**S_{10}**	
P_1	0	0	1	0	0	0	1	1	0	1	1
P_2	1	1	0	1	0	0	0	0	0	1	1
P_3	0	0	1	1	1	0	0	0	1	0	0
P_4	1	0	0	0	1	1	0	1	0	0	1
P_5	0	1	0	0	0	1	1	0	1	0	0
P_6	1	1	1	1	1	1	1	1	1	1	1

Scenario 3. The pooling results in one potential positive sample

Pooled samples	Original samples										Logical value
	S_1	**S_2**	**S_3**	**S_4**	**S_5**	**S_6**	**S_7**	**S_8**	**S_9**	**S_{10}**	
P_1	0	0	1	0	0	0	1	1	0	1	0
P_2	1	1	0	1	0	0	0	0	0	1	0
P_3	0	0	1	1	1	0	0	0	1	0	1
P_4	1	0	0	0	1	1	0	1	0	0	1
P_5	0	1	0	0	0	1	1	0	1	0	0
P_6	1	1	1	1	1	1	1	1	1	1	1

'0' negative, '1'positive.
Original samples in black bold (**S_i**) with '1' in blue below are logically discarded in each pool.
Original samples in red bold (**S_i**) not discarded in any pool must be individually tested.

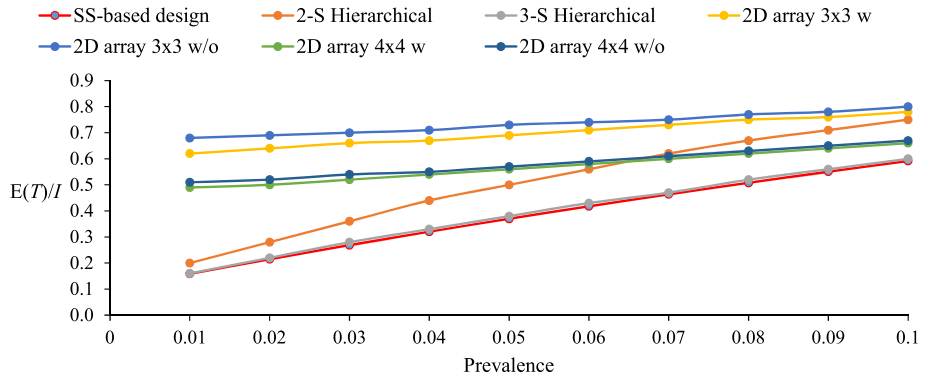

Fig. 1. Efficiency of pooling algorithms for different prevalence values *p* ranging from 0.01 to 0.10. Expected number of tests per individual sample E(*T*)/*I*.

pool, is not as sensitive to variations in the prevalence rate. Then, even without having been optimized, the proposed procedure achieves unambiguous identification and greater efficiency than other methods, especially for low prevalence rates.

When the analytical test is not perfectly accurate, the computation of the efficiency of any pooling strategy should consider the sensitivity (Sens) and specificity (Spec) of the analytical test. Sens is the probability that a truly positive sample will be considered as positive, and Spec is the probability that a truly negative sample will be considered as negative. Both are assumed to be independent of pool size, so pooling

samples does not alter the likelihood of identifying positive cases or the false negative rate, i.e., there is no dilution effect, which requires prior knowledge of the maximum pool size for a particular application.

A comparative analysis of efficiency for various pairs Sens/Spec of the analytical test used in the screening method is shown in Fig. 2. A detailed explanation of the calculations for each algorithm can be found in [30].

The proposed pooling algorithm, supersaturated-based design (red line), reaches the lowest values of E(T)/I, and thus the highest efficiency, the closest being the 3-S hierarchical with the Optimal Test Configuration (OTC), i.e., a first pool size of 10 and pool sizes of 4, 3, and 3 in the second stage. Whatever the pooling algorithm, an asymmetric effect on efficiency of Sens and Spec of the analytical test is seen. Comparison between Sens/Spec pairs 0.99/0.90 and 0.90/0.99 reveals lower efficiency (higher values of E(T)/I) for low Spec of the analytical test (0.99/0.90). A low Sens of the analytical test (0.90/0.99) has less influence on efficiency.

2.4.2. Pooling sensitivity and specificity

In addition to efficiency, the accuracy of correctly classifying truly positive and negative samples must be considered. The 'pooling sensitivity', PS_e, is the probability of a correct positive diagnosis after the pooling procedure has been applied to the i-th sample. Similarly, the 'pooling specificity', PS_p, is the probability of a correct negative diagnosis.

Given a prevalence $p = 0.05$, PS_e and PS_p have been computed, following [30], for different pairs of Sens/Spec of the analytical test used in the screening (Figs. 3 and 4, respectively).

PS_e of our proposal (line in red) falls when there is a low Sens (0.90) of the analytical test, particularly when compared to the 2D array w/master pool algorithm, which works with a large master pool (121 samples). Nevertheless, it should be noted that 2D array algorithms have a much lower efficiency (as seen in Fig. 1).

Regarding the Pooling Specificity (Fig. 4), the pooling procedure suggested here (line in red) shows higher values of PS_p for any pair Sens/Spec of the analytical test, compared to the remaining algorithms.

A feature shared by all the pooling algorithms (except the 2D array with master pool of 121 samples) is that Sens and Spec of the analytical test have an asymmetric impact on PS_e and PS_p. Comparing pairs 0.99/0.90 and 0.90/0.99 in both Figs. 3 and 4, for a low Sens (0.90), there is a remarkable decrease in PS_e (Fig. 3) but PS_p (Fig. 4) does not experience that drop. On the contrary, except for the 2D array w/master pool, if Spec is low (0.90), PS_e and PS_p are not affected.

On the other hand, there is a great similarity between the results of the proposed algorithm and those of the 3-S hierarchical when the optimal configuration is considered. Numerical differences between SS-based design and 3-S hierarchical are so small that are not visible in either Fig. 3 or Fig. 4. The proposed algorithm, supersaturated-based design, works with an initial sample of size 10, and neither the design matrix structure nor the pooled sample size is optimized.

3. Software and experimental

To perform the procedure explained in Section 2.3 a function, *solver1*, written in MATLAB [37] whose code can be found in https://hdl.handle.net/10259/7757, has been used.

The methodology suggested for obtaining the pooled samples and the identification of the true positive samples has been applied to the detection of the pathogen *Salmonella spp* and detection of nut DNA by polymerase chain reaction (PCR). The presence of *Salmonella* is regulated by Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. As for nuts, it appears as one the products causing allergies and intolerances in Regulation (EU) No 1169/2011 [11].

The laboratory AGROLAB IBERICA, S.L.U. Burgos, has carried out the PCR analyses by accredited method (Standard UNE-EN ISO/IEC 17,025 accreditation n°258/LE2068). The iQ-Check Salmonella II Kit (supplied by Bio Rad, Madrid, Spain) has been used. This kit is certified by AFNOR (reference BRD 07/06—07/04) as an alternative to the reference method ISO 6579—1 (2017) for the detection of *Salmonella spp,* while the kit SureFood® ALLERGEN Walnut" de R-Biopharm (Darmstadt, Germany) has been used for walnuts.

The samples have been prepared using two food matrices: rice flour and liquid sample (with carbohydrates as the main component). Four batches, each with ten samples, have been prepared with each of food matrices. When liquid samples with carbohydrates as the main component were used, one, two or no samples have been doped with *Salmonella.* Similarly, when rice flour matrix was used, one, two or no samples have been doped with nuts. In the first batch, one sample was randomly chosen to be doped. The second batch had two doped samples, randomly chosen out of the 30 possibilities involving the analysis of 3 individual samples in step 3. Similarly, two samples were doped in the third batch, but randomly chosen from 15 possibilities involving the analysis of 6 individual samples in step 3. Finally, no samples were doped in the fourth batch.

According to the methodology suggested, the samples to be individually tested are determined once the outcomes of step 2 are available. Therefore, the preparation of the doped samples has been performed by a third party, independent of AGROLAB analysts as well as researchers, that is, following a double-blind protocol, identical for *Salmonella* and *nut* allergens. The four batches were sent in random order to the laboratory, which did not know the number and position in the batch of the

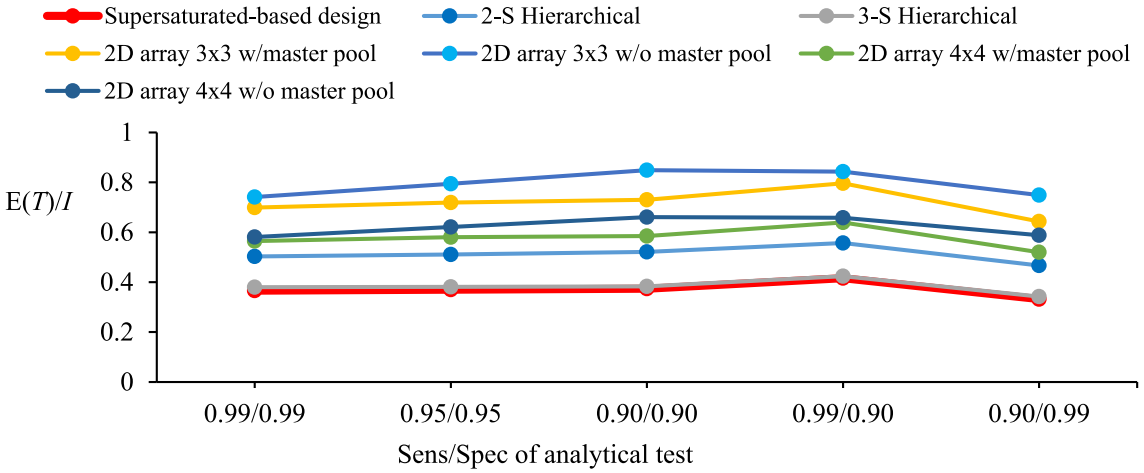

Fig. 2. Efficiency versus Sens/Spec of the analytical test for different pooling algorithms.

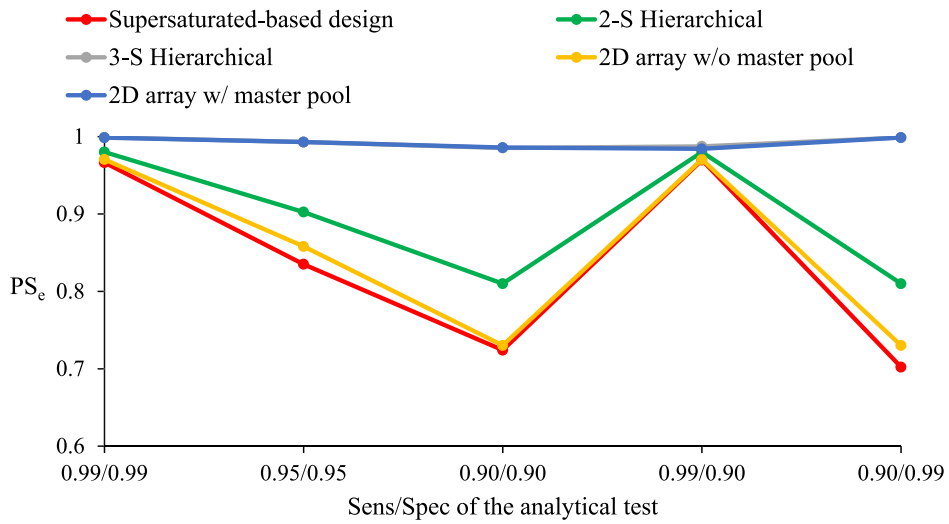

Fig. 3. Pooling Sensitivity (PS_e) according to Sens/Spec of the analytical test.

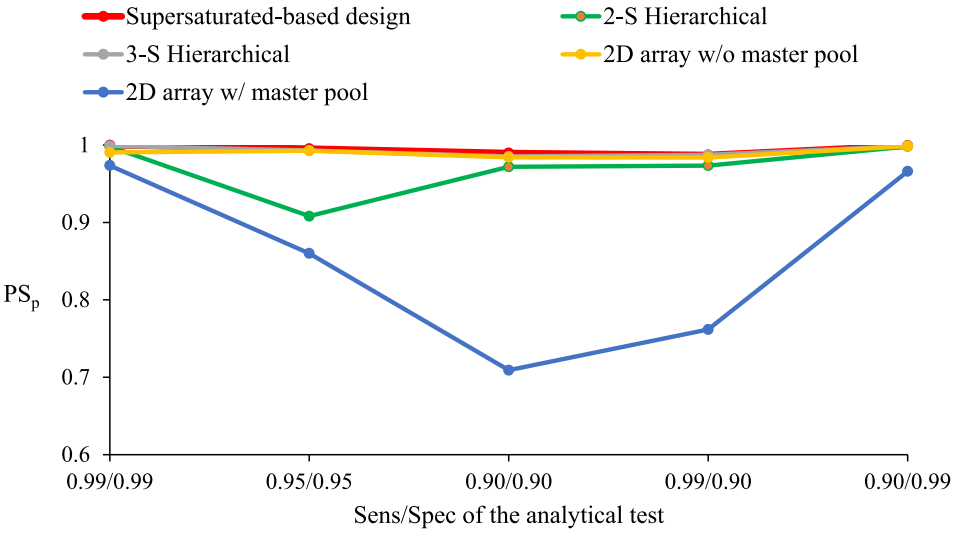

Fig. 4. Pooling Specificity (PS_p) according to Sens/Spec of the analytical test.

doped samples. The laboratory made two aliquots of each individual sample, using one of them to prepare the pooled samples of each batch following Table 1, and then sent the results. After applying the proposed method (step 2), the laboratory performed PCR analyses of the potential positive samples in each batch (step 3) and presented the final results.

4. Screening for *salmonella* and nuts in food samples using the proposed pooling strategy

4.1. Identification of doped samples

The suggested procedure has been applied to the screening of a food pathogen (*salmonella spp*) and an allergen (nut). The detection of *salmonella* leads to the results listed in Table 2. The four cases discussed here are based on the pooling design outlined by the the half-fraction of a PB matrix in Table S1 (in the supplementary material) and the pooling procedure detailed in section 2.2. Remind that the sixth pooled sample in this design matrix, P_6, corresponds to the 'master pool' and must be tested in the first step of the pooling procedure described.

As Table 2a shows, the PCR of the 'master pool' in Case IV is negative, so all samples are negative, and no added steps are needed. However, Case I results in only one negative outcome, P_2, which allows discarding four samples and the remaining six, S_3, S_5, S_6, S_7 . S_8 . S_9, are potential positive and must be retested to find out the true positive ones: S_5 . S_7. As to Case III, since two pooled samples test negative, more samples can be discarded, so only S_1 . S_8 . S_{10} need to be retested to identify the true positive ones: S_8, S_{10}. Finally, the pooling is particularly beneficial in Case II, with only two positive pooled samples, P_2 P_5, pointing to S_2, which is proved positive by individual testing.

Likewise, the identification of the samples contaminated with an allergen (nut) follows the procedure summarized in Table 2b as mentioned, no further test is needed when the 'master pool' is negative, as in Case II. Otherwise, the more pooled samples testing positive in Step 2, the more individual tests will be required in Step 3. For example, in Case IV, all the pooled samples but one, have a positive PCR, so only a few original samples are discarded and the remaining six candidates, S_1, S_2 . S_4 S_5 . S_6 . S_9, must be retested to detect the true positive ones, S_1 and S_9. The pooling is more advantageous in Case I, given that two negative pooled samples lead to three potential positive samples (S_5 . S_6 . S_9), where the first two are finally identified. Finally, the pooling is a profitable solution in Case III, which directly targets the only contaminated sample, S_{10}.

Therefore, using the suggested pooling design and the logical methodology, all samples doped with either *salmonella spp* or nut have

Table 2
a) detection of *Salmonella* and b) *nut* allergen by means of PCR and logical solution for the identification of positive samples. '1′ denotes a positive outcome, and '0′, a negative outcome. The candidates determined in Steps 1 and 2 are individually tested in Step 3.

a) *salmonella*		Case I	Case II	Case III	Case IV
Step 1	Master pool (P_6)	1	1	1	0
Step 2	P_1	1	0	1	–
	P_2	0	1	1	
	P_3	1	0	0	
	P_4	1	0	1	
	P_5	1	1	0	
Step 3	Potential positive	S_3 S_5 S_6 S_7 S_8 S_9	S_2	S_1 S_8 S_{10}	–
	True positive	S_5 S_7	S_2	S_8 S_{10}	–

b) nut		Case I	Case II	Case III	Case IV
Step 1	Master pool (P_6)	1	0	1	1
Step 2	P_1	0	–	1	0
	P_2	0		1	1
	P_3	1		0	1
	P_4	1		0	1
	P_5	1		0	1
Step 3	Potential positive	S_5 S_6 S_9	–	S_{10}	S_1 S_2 S_4 S_5 S_6 S_9
	True positive	S_5 S_6	–	S_{10}	S_1 S_9

been correctly detected.

4.2. Efficiency of SS-based pooling strategy for salmonella spp and nuts

As mentioned in the introduction of this paper, the detection of zoonoses and allergens has become a priority for health authorities, and therefore screening methods on food samples are consistently implemented. Some of the analytics on food safety policy in the EU will be used here to assess the pooling strategy applied to this work. Regarding allergens, no official EU reports have been released on the number of samples evaluated or their breakdown by allergen. For the reported overall prevalence of 0.131, included in the introduction of this work, the application of the SS-based pooling results in an efficiency of 0.71 (95% CI 0.64–0.77). Using a similar sample size, this means a 29% reduction in the number of analytical determinations (95% CI 23%–33%).

Conversely, the *salmonella* monitoring in the EU is exhaustively described in ref. [6]. As regards the verification of the food safety criteria for Salmonella in accordance with Regulation (EC) No 2073/2005, the reported number of tested samples is grouped by: 1) the stage in the food chain, manufacturing or distribution, 2) different species of animal carcasses, distinguishing the monitoring carried out by competent authority (CA) from those carried out by food business operators (FBOp), 3) other samples categorized as ready-to-eat (RTE) and non-ready-to-eat (non-RTE) food.

Although this report does not specify the analytical procedure followed in each determination, it always leads to a binary outcome: contaminated/ uncontaminated sample, and therefore the SS-based pooling strategy used in this paper could be implemented. In this regard, a comparison using data on the number of samples tested required at EU level in 2021 makes sense. Anyway, the calculation of the efficiency of the SS-based pooling strategy, from the number of tested samples and the prevalence estimated in the EU in 2021, assumes the use of a specific pooling size. If certain samples had to be analyzed individually, confirmatory analyses or unacceptable waiting times to complete pools of 10 samples, a proportion of the total amount of samples registered by EFSA in 2021 would not have been part of any size 10 pool, and thus those samples would be out of comparison.

For $S_e = S_p = 1$, results for the criteria used in the EFSA report are shown in Tables S3 to S5 of the supplementary material. In 2021, the total amount of samples registered by EFSA was 1,051,442. As they were presumably analyzed individually, this means the same number of analytical tests. If the SS-based pooling had been applied to that number of samples, the expected number of analytical tests would have been 204,570, thus implying a significant cost reduction or, alternatively, an increase in the number of samples inspected at the same cost. Furthermore, it should be noted that the SS-based pooling method is designed to analytically identify positive samples, i.e., the result 'positive sample' does not come from any calculation derived from the experimental outcomes but is determined by the analytical test.

As to the different variables considered, Table S3 of the supplementary material shows *salmonella* positive samples from official sampling either at the manufacturing or the distribution stage. The reduction is similar in both cases, a bit lower in the distribution stage going from 22,614 to 5,475 analyses.

As regards the monitoring of carcasses of distinct species, Table S4 of the supplementary shows a reduction in the number of analyses from 50,479 to 13,103 when conducted by the competent authority, which is lower than the reduction in analyses conducted by the food business operator, going from 406,609 to 66,627 analyses. This is because, except for the control of goat carcasses, the prevalence is always higher with the data of the competent authority.

A comparison of RTE, ready-to-eat, with non-RTE, non-ready-to-eat food (Table S5 of the supplementary) reveals a lower prevalence in the former, and therefore a greater efficiency if the SS-based pooling strategy were implemented, which would reduce the number of analytical tests from 73,238 to 8,327.

The EFSA report does not provide the sensitivity and specificity of the analytical methods used, but they can reasonably vary between 0.90 and 0.99. That is why two additional scenarios have been considered for the study of efficiency, E(T)/I, as a function of the sensitivity and specificity of the analytical screening method: the first one being $S_e = 0.90$ and $S_p = 0.99$, and the second one with $S_e = 0.99$ and $S_p = 0.90$, both included in Tables S6–S8 of the supplementary material. According to the information shown in Fig. 2, Sens/Spec 0.99/0.90 has the lowest efficiency while Sens/Spec 0.90/0.99 is the most efficient.

Taking a close look at Tables S6–S8, where S_e and S_p are different from 1, some conclusions can be drawn about the efficiency of the SS-based pooling versus individual sampling.

First of all, analytical tests with $S_e = 0.90$ and $S_p = 0.99$ provide greater efficiency gains than those with $S_e = 0.99$ and $S_p = 0.90$, confirming the asymmetric impact of sensitivity and specificity on the expected number of analyses. As showed in Table S6, the greatest improvement in efficiency occurs when specificity equals 0.99, i.e., in the first scenario (Sens/Spec is 0.90/0.99), for samples contaminated with *salmonella* both in the manufacturing and in the distribution stage. For example, analyses would fall from 22,614 to 5,070 if Sens/Spec is 0.90/0.99 versus a drop of up to 6,610 in the second scenario (Sens/Spec equal to 0.99/0.90). A similar pattern can be observed in Table S8, where contaminated samples in RTE and non-RTE food are compared. In this case the number analyses would fall from 73,238 to 8,518 (versus 12,201 when Sens/Spec is 0.99/0.90).

An analogous idea is revealed by Table S7 with data on contaminated samples from carcasses of distinct species. As it has already been noticed, species with low prevalence of *salmonella* (say less than 1%) such as bovine cattle, goat or horse samples show large efficiency gains (Eff(SS) values close to 0.14 with data from the competent authority), while broiler and turkey samples, with a prevalence well over 5%, would benefit from the pooling strategy used to a lesser extent. Anyway, the effect of the accuracy of the analytical test follows a similar pattern: efficiency gains are greater when Sens/Spec is 0.90/0.99.

Secondly, the differences regarding efficiency gains between the two Sens/Spec scenarios are larger when *salmonella* prevalence is high.

Focusing on broiler samples taken by the competent authority (prevalence of 14%), values of Eff(SS) are 0.6150 (first scenario) versus 0.7521 (second scenario), so improvements in efficiency induced by the SS-based pooling are particularly reinforced when the Sens/Spec pair equals 0.90/0.99. It is worth mentioning that when broiler samples are taken by the food business operators, a prevalence of 3.2% is estimated, and the scenario regarding the accuracy of the analytical test, Sens/Spec pair, has less effect on efficiency gains (0.2582 for 0.90/0.99, versus 0.3272 for 0.99/0.90). The same pattern can be drawn from data of turkey samples.

In summary, the sensitivity and specificity of the analytical method play a relevant role when applying sample pooling methods, particularly for the SS-based pooling strategy.

5. Conclusion

The pooling procedure used improves the performance of existing pooling algorithms in terms of efficiency, while maintaining accuracy, sensitivity, and specificity, and is a suitable procedure for the detection of *salmonella spp* and nut allergens.

The number of tests per individual sample is expected to range between 0.10, for a prevalence below 1%, and 0.59, for a prevalence above 10%.

Pooling sensitivity (PS_e) and pooling specificity (PS_p) depend on pairs Sens/Spec of the analytical test asymmetrically. For the strategy proposed, PS_p ranges from 0.9872 to 0.9999, provided that Sens and Spec are equal to or greater than 0.90. Conversely, if Sens of the analytical test is below 0.99, PS_e does not reach the maximum values. The best results for PS_e (0.9695 and 0.9697) occur when Sens/Spec are 0.99/0.99 and 0.99/0.90 respectively.

Using the latest reported data on *salmonella* monitoring in the EU, calculations have been made on the expected efficiency gains induced by the SS-based pooling strategy for this particular pathogen, estimating large improvements over individual sampling. The estimates have been broken down by the stage in the food chain, manufacturing or distribution, the distinct species of animal carcasses supervised by the competent authority and by the food business operators, and the categorization of samples as ready-to-eat (RTE) and non-ready-to-eat (non-RTE) food.

The pooling procedure used can be automized for systematic use in screening by PCR of pathogens, particularly *salmonella*, and food allergens, as well as generalized to other qualitative analysis procedures. A pooling of size 10 is not difficult to implement in the field of food safety compared to, for example, a 2D hierarchical pooling of size 100.

This strategy could increase the testing capacity of laboratories, saving time and resources, so that screening of foodborne pathogens and allergens becomes more sustainable.

CRediT authorship contribution statement

O. Valencia: Investigation, Data curation, Writing – original draft. **L. A. Sarabia:** Data curation, Conceptualization, Methodology, Software, Writing – review & editing. **M.C. Ortiz:** Data curation, Visualization, Supervision, Validation, Funding acquisition.

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.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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Appendix A. Supplementary data

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