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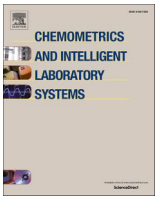

Logical analysis of sample pooling for qualitative analytical testing

L.A. Sarabia [a], O. Valencia [a], M.C. Ortiz [b,*]

[a] Departamento de Matemáticas y Computación, Universidad de Burgps. Facultad de Ciencias, Plaza Misael Bañuelos s/n, 09001, Burgos, Spain
[b] Departamento de Química, Universidad de Burgos, Facultad de Ciencias, Plaza Misael Bañuelos s/n, 09001, Burgos, Spain

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ABSTRACT

When the prevalence of positive samples in a whole population is low, the pooling of samples to detect them has been widely used for epidemic control. However, its usefulness for applying analytical screening procedures in food safety (microbiological or allergen control), fraud detection or environmental monitoring is also evident.

The expected number of tests per individual sample that is necessary to identify all 'positives' is a measure of the efficiency of a sample pooling strategy. Reducing this figure is key to an effective use of available resources in environmental control and food safety. This reduction becomes critical when the availability of analytical tests is limited, as the SARS-CoV-2 pandemic showed.

The outcome of the qualitative analytical test is binary. Therefore, the operation governing the outcome of the pooled samples is not an algebraic sum of the individual results but the logical operator ' $\vee$ ' ('or' in natural language). Consequently, the problem of using pooled samples to identify positive samples naturally leads to proposing a system of logical equations. Therefore, this work suggests a new strategy of sample pooling based on: i) A half-fraction of a Placket-Burman design to make the pooled samples and ii) The logical resolution, not numerical, to identify the positive samples from the outcomes of the analysis of the pooled samples.

For a prevalence of 'positive' equal to 0.05 and 10 original samples to be pooled, the algorithm presented here results in an expected value per individual equal to 0.37, meaning a 63% reduction in the expected number of tests per individual sample.

With sensitivities and specificities of the analytical test ranging from 0.90 to 0.99, the expected number of tests per individual ranges from 0.332 to 0.416, always higher than other pooled testing algorithms. In addition, the accuracy of the algorithm proposed is better or similar to that of other published algorithms, with an expected number of hits ranging from 99.16 to 99.90%.

The procedure is applied to the detection of food samples contaminated with a pathogen (*Listeria monocytogenes*) and others contaminated with an allergen (*Pistachio*) by means of Polymerase Chain Reaction, PCR, test.

1. Introduction

Pooled testing or 'group testing' is the process of testing samples together as a combined group, rather than individually, to identify those samples with a binary trait of interest: positive samples. When a 'pooled sample' (group) tests negative, none of the original samples included in it needs to be analysed so all of them are discarded. If the pooled sample tests positive, at least one of its original samples is positive so further investigation is required. This might involve an individual analysis of each original sample or additional steps of sample pooling to unambiguously identify the positive original samples. As long as the prevalence of the trait of interest is small and appropriate group sizes are chosen, pooled testing can lead to substantial reductions in the number of tests when compared to testing each sample individually.

The pioneer work in group testing emerges from Dorfman's research on the detection of defective members in large populations [1], since procedures based on individual inspections, whether a rare infectious disease in a population or defective items in manufacturing, are often time-consuming and unaffordable.

Although Dorfman's method has been widely used in medical applications, further developments in group testing have led to increasing testing capacity which has benefited testing a series of diseases in a variety of human applications, including blood donation screening [2], detection for Human Immunodeficiency Virus, HIV [3–5], influenza

* Corresponding author.
E-mail address: mcortiz@ubu.es (M.C. Ortiz).

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outbreak surveillance [6,7], chlamydia and gonorrhea testing [8], detection of Hepatitis C virus, HVC [9], drug screening [10] as well as veterinarian applications, such as salmonella in horses [11], disease detection in cattle and buffaloes [12], West Nile virus in mosquitoes [13], trichomoniasis in cattle [14] or animal testing of multiple diseases [15].

In addition to biomedical studies, group testing has also been employed in a large number of applications in environmental studies (pesticides in water) [16], trace metals in water [17], human exposure to pollutants [18], and food safety (dioxin monitoring in milk [19], bovine milk surveillance [20], salmonella in eggs [21], salmonella in pigs [22], salmonella in birds [23], surveillance in aquaculture [24]).

Recently, sample pooling has been largely boosted by the Severe Acute Respiratory Syndrome CoronaVirus 2, SARS-CoV-2, pandemic [25,26], an unprecedented public health crisis. The shortage of Polymerase Chain Reaction, PCR, assays, the gold standard for identifying infection required, coupled with the fast spread of emerging variants, requires rapid diagnostic and screening tests and has prompted research to increase the testing capacity of laboratories. A search in Scopus with the terms 'Group testing' or 'Pooled testing' or 'Sample pooling' or 'Specimen pooling' within title-keywords provides more than eight hundred document results, 248 of them published since 2020. Of the latter, 180 contain the term 'SARS-CoV-2' or 'COVID-19' and 189 include the term 'PCR'. The literature on sample pooling related to the SARS-CoV-2 has highlighted the growth in testing capacity and the drastic reduction in testing times needed, by means of different pooling methodologies aimed at determining, among other issues, the optimal number of samples that could successfully be combined into a pool for a given prevalence [27–31].

The mathematical theory on group testing can be found in Ref. [32]. Given the total number of samples and the number of positive samples, the aim is to figure out the number of groups (tests) needed to accurately identify the positive samples, and the composition of these groups. An extensive survey of recent developments for group testing can be found in Ref. [33], including a classification of algorithms based on different criteria: adaptive (groups designed sequentially depending on previous test outcomes) *vs* nonadaptive, noiseless (the test procedure works perfectly) *vs* noisy testing, exact recovery (all positive samples are detected) *vs* partial recovery, binary *vs* non-binary outcomes.

According to Ref. [33], the statistical aspects of group testing are typically broken up into two different problems. The first one is the identification problem, where the aim is developing an algorithm which minimizes the expected number of tests per individual sample while keeping the expected accuracy as high as possible, which implies the optimal group size selection [34]. The second one is the estimation problem, aiming at estimation of prevalence (the overall proportion of positive samples) or of individual specific probability of being positive as function of a set of given factors. This paper is focused on the identification problem.

The assessment of group testing algorithms can be conducted with some figures or merit or operational characteristics. The most commonly used is the efficiency or expected number of tests per individual. However, in the presence of test error, accuracy of outcomes should be considered. This entails the computation of four additional figures of merit: the pooling sensitivity, that is, the probability that a truly positive original sample will be categorized as positive, the pooling specificity, i.e, the probability that a truly negative original sample will be categorized as negative, as well as the Positive Predictive Value(PPV) which is the probability that a primary positive will be a true positive and the Negative Predictive Value (NPP) or probability that a primary negative will be a true negative.

Denoting by T, the number of tests required to identify the positive samples for an initial group of size I, some assumptions are made to determine the efficiency [35]. Firstly, all original samples are independent and identically distributed with probability p of being positive, so prevalence equals p. Secondly, given a pooled sample having at least one positive original sample, the probability of testing positive S_e (test sensitivity) equals the sensitivity for a test of an individual sample, that is a pool of size 1. Finally, for a pooled sample with no 'positives', the probability of testing positive is equal to 1-S_p, where S_p is the test specificity, which is assumed to be independent of pool size. The last two imply that there is no dilution effect, i.e. that pooling samples does not alter the likelihood of identifying positive cases or the false negative rate, thus requiring a prior knowledge of the maximum pool size for a particular application. Models incorporating dilution effects may be found in Refs. [36,37].

Group testing algorithms are usually classified into hierarchical and non-hierarchical categories [33,38]. In hierarchical group testing, individuals are tested over multiple stages in non-overlapping groups. The original idea of Dorfman is a two-stage algorithm, but three-stage and four-stage algorithms are also frequently used [39]. As to non-hierarchical procedures (non-adaptative group testing), individual samples are tested in overlapping groups at the initial stage, so an individual sample can be represented in more than a group, with the final purpose to reduce the number of individual samples which have to go to a further stage, i. e., the number of retests.

In 2D-array testing, all original samples, I, 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. After analyzing the pooled samples, an individual sample in cell (i, j) must be retested in a second stage if row i tests positive and column j tests positive [40]. Array testing may also involve initially testing a 'master group' consisting of all samples within the array. If this group tests negative, all samples are declared negative. Otherwise, the testing of row and column groups proceeds as mentioned. When individual samples have unequal probabilities of being positive, the configuration of a 2D-array can be modified to isolate the higher-risk samples using gradient or spiral methods [41], thus expecting as negative most of rows and columns.

Instead of 2D-arrays, samples can be arranged either physically or algorithmically into a 3D cubical structure, where groups are formed over rows, columns, and layers, and intersections of positive groups show the possibility of positive simples [42].

Traditionally, group testing assumes the same probability of being positive for all samples, which is often unrealistic. When additional information (known risk factors) is available on samples, the probability of being positive may vary from sample to sample, so the testing process should consider heterogeneous probabilities to reinforce its efficiency. Recent research on the so-called Informative group testing, also known as risk-based pooling or generalized group testing, has been performed [43,44]. In hierarchical algorithms, this results in testing low probability samples in large groups while high risk samples are tested in small groups. In array testing, samples are arranged in the array to minimize the number of rows and columns that tests positive. The implementation of informative group testing requires the estimation of these probabilities, which may be performed using regression models.

Taking into account the group testing algorithm to be implemented, an optimal set of group sizes, the so-called optimal testing configuration (OTC), has to be determined. To do this, a function must be selected and optimized. As suggested, the most common objective function is efficiency, so from I original samples, represented within one initial group for hierarchical testing or in one array for array testing, the expected number or tests per individual sample, $E(T)/I$ is minimized. However, alternative functions including accuracy, that is the expected number of correct classifications C, can be used. This results in maximizing accuracy per individual sample divided by the expected number of tests per individual sample, $E(C)/E(T)$, or equivalently, minimizing $E(T)/E(C)$ [45]. A different pooling strategy to get the optimal pooled samples is built in Ref. [46] through a Bayesian D-optimal experimental design, where the function to maximize is the mutual information between the binary response (negative/positive) of the pooled sample and the code assigned according to the individual samples pooled, which is equivalent to minimizing the expected posterior entropy.

Optimal group testing strategies have also been developed when testing for more than one response variable at a time [47], for example, in screening for sexually transmitted diseases, or for coronavirus and other viruses producing similar symptoms. The R package binGroup2, Statistical Tools for Infection Identification via Group Testing, encompasses 27 different algorithms for the usual setting of a binary response (positive/negative) from independent individuals within a group. They allow testing error and single or multiple-response assays, including hierarchical/non-hierarchical algorithms in homogeneous or heterogeneous populations. The optimal testing configuration is based on an estimated prevalence, a test's reliability in detecting positive and negative cases and a range of potential initial pool sizes [48].

Within the field of chemometrics, some authors [49–51] have developed a different approach to obtain the pooled samples in the analytical laboratory by means of supersaturated (SS) designs of experiments.

These kinds of designs are suitable for screening when the number of potential factors is very high, thus giving place to an unfeasible number of experimental units in terms of both costs and time. SS designs are characterized by N_{ss} experimental units to study $f_{ss} \geq N_{ss}$-1 factors. Therefore, there are not enough degrees of freedom to properly estimate the factors main effects, which might be somehow confused with some interactions. As perfect orthogonality is impossible to achieve, SS designs are built by using an optimality criterion which approaches orthogonality as much as possible.

Nevertheless, in the early exploratory stages of an investigation, when the aim is to identify the critical factors, disregarding interactions which will be better detected in later stages, some SS designs have been proved very useful [52].

In the context of sample pooling, or what the research group of Cela calls Strategic Sample Composition (SSC), the factors (columns in the SS design matrix) are the original samples from which the pooling is made, whereas the experimental units are the pooled samples (rows in the SS design matrix). As each original sample may be either absent (−1) or present (+1) in a particular pooled sample, we are dealing with 2-level designs.

Regarding the pooling of samples, SS designs make sense because the interactions effects are actually null, i.e., effects are additive in the analytical assay, resulting in a small number of experimental units (pooled samples).

In this work, a new sample pooling algorithm is suggested. It is based on a SS design, particularly on a half-fraction of a Plackett-Burman design with N rows and f columns, using a branching column. This results in a procedure that is developed in several stages but cannot be categorized as hierarchical since the pooled samples overlap.

The distinctive feature of qualitative assays, such as PCR, is that the experimental response of the assay is binary (negative/positive). Therefore, whether a pooled sample includes one or more positive samples, the result remains just as positive regardless of the number of positive samples that form it. Consequently, unlike other applications of SS designs, the analysis of the results is not obtained by regressing the analytical response of the pooled sample (0/1) on the code assigned to the pooled sample according to the individual samples pooled.

This paper is organized as follows: After this introduction, the proposed methodology is formally described in section 2. This section includes the design of the pooled samples (section 2.1), the logical formalization of sample pooling, and the determination of the original samples to be analysed individually (sections 2.2 and 2.3). This section ends with an illustrative example of the methodology (section 2.4). Section 3 details the software, analytical method, and preparation of the original samples to which the proposed methodology will be applied. The results and discussion are in Section 4, which includes the efficiency and accuracy of the proposed pooling method, and their comparison with other published algorithms, as well as the application to the detection of the pathogen *Listeria monocytogenes* and the allergen *Pistachio*.

Finally, the paper ends with some conclusions, several references and two annexes, one with a MATLAB function to solve the logical system, and the other with the calculations for the expected number of tests.

2. Methodology

This section describes: i) the procedure to obtain the pooled samples, ii) the logical modeling of the problem, and iii) the logical procedure leading to the identification of the original positive samples.

2.1. Experimental design

Regarding the procedure to get the pooled samples from I original samples, let T be the number of tests performed on pooled samples. The test matrix (or test design) is denoted by $\mathbf{X} = (x_{ti}), t = 1, 2, \ldots, T, i = 1, 2, \ldots, I$ where $x_{ti} = 1$ if the i-th original sample is in the t-th pooled sample and $x_{ti} = 0$ if the i-th original sample is not in the t-th pooled sample.

Instead of using a traditional pooling algorithm (hierarchical or array-wise), the pooled samples have been generated using a SS design, built in turn as a half-fraction of a Plackett-Burman design with N rows and f columns PB(N,f). Following [53], one column of the PB(N,f) is chosen as branching column so that all experiments where the branching column is either at high or low level, respectively, are selected and then the branching column is deleted, resulting in two supersaturated designs with $f_{SS} = f-1$ factors and $N_{SS} = N/2$ experiments. Then, the half-fraction containing the experiments with all factors at level (−1) is retained. Therefore, each row represents a pooled sample formed by the original samples that correspond to the code (−1) of the retained half-fraction. Particularly, a half-fraction of a PB(12,11) design is used, so we are dealing with $f_{SS} = 11-1$ factors, the $i = 10$ original samples, and a total $N_{SS} = 12/2$ experiments, the $T = 6$ pooled samples to be analysed.

To adapt the notation of the design matrix to the traditional notation of pooled testing, the usual '-1' of the PB matrix has been replaced with '1', and the '+1' of the PB with '0'. The design matrix, $\mathbf{X}_{6\times10}$, is shown in Table 1, where the elements $x_{ti} = 1$ indicate the original samples S_i, $i = 1, \ldots, 10$ (columns) which are combined in each pooled sample P_t, $t = 1, \ldots, 6$ (rows).

2.2. Logical modeling of the problem

The application of an analytical test, such as a PCR, to the i-th original sample S_i, $i = 1, \ldots, I$, results in a 'logical value', $lv(S_i)$, to the statement 'the sample S_i is positive'. This is formally defined in Eq. (1)

$$lv(S_i) = \begin{cases} 0, \text{if the statement 'the original sample } S_i \text{ is positive' is false} \\ 1, \text{if the statement 'the original sample } S_i \text{ is positive' is true} \end{cases} \quad (1)$$

The same definition applies to each pooled sample P_t, $t = 1,2, \ldots, T$. Formally defined in Eq. (2)

$$lv(P_t) = \begin{cases} 0, \text{if the statement 'the pooled sample } P_t \text{ is positive' is false} \\ 1, \text{if the statement 'the pooled sample } P_t \text{ is positive' is true} \end{cases} \quad (2)$$

In the problem studied here, once the tests (e.g. PCR tests) have been

Table 1
Design matrix to obtain the pooled samples, built with a half-fraction of a Plackett-Burman (12,11). The '1' represents the original samples included in each of the six pooled samples used.

Pooled samples	Original samples									
	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
P_2	1	1	0	1	0	0	0	0	0	1
P_3	0	0	1	1	1	0	0	0	1	0
P_4	1	0	0	0	1	1	0	1	0	0
P_5	0	1	0	0	0	1	1	0	1	0
P_6	1	1	1	1	1	1	1	1	1	1

performed, the $lv(\mathrm{P_t})$ are known experimentally, while the $lv(\mathrm{S_i})$ are the unknowns to be found.

From the design matrix **X**, using the Boolean inclusive OR operator $\vee$, where $0 \vee 0 = 0$ and $0 \vee 1 = 1 \vee 0 = 1 \vee 1 = 1$, the formal equations of the logical values are constructed. There is one equation for each pooled sample, which reflects its distinctive composition and its test outcome. Formally, we have the system, Eq. (3), of the T logical equations:

$$lv(\mathrm{P_t}) = \bigvee_{i=1}^{I} x_{ti} lv(\mathrm{S_i}), t = 1, 2, \ldots, T \tag{3}$$

As usually denoted, let $y_t \in \{0, 1\}$ be the outcome of the test on the t-th pooled sample, $t = 1, 2, \ldots, T$. Assuming a noiseless framework, i.e., both sensitivity and specificity equal to 1 as well as no dilution effect, $y_t = 1$ (positive outcome in a pooled sample) if at least one of the original samples is positive, and $y_t = 0$ (negative outcome in a pooled sample) if all the original samples are negative. Therefore, $y_t = lv(\mathrm{P_t})$ and the system of Eq. (3) becomes:

$$y_t = \bigvee_{i=1}^{I} x_{ti} lv(\mathrm{S_i}), t = 1, 2, \ldots, T \tag{4}$$

In the system of logical Eq. (4), both the coefficients $x_{ti}, t = 1, 2, \ldots, T, i = 1, 2, \ldots I$ forming the design matrix **X**, and the outcomes of the pooled samples, $y_t, t = 1, 2, \ldots, T$, are known, whereas the logical values identifying the positive original samples $lv(\mathrm{S_i}), i = 1, 2, \ldots, I$ have to be found.

This formulation of the problem prevents the use of numerical methods to solve systems of algebraic equations and requires a logical solution.

2.3. Logical procedure for the identification of positive samples

The logical method for unequivocally identifying the positive original samples is developed in the following three rounds:

Round 1: First, the pooled sample number 6 of Table 1, formed by all original samples or 'master pool', is analysed. If it tests negative, then all the original samples are negative, otherwise there is at least one positive sample and we have to move on to round 2.

Round 2: T-1, that is, the remaining 5 pooled samples are analysed.

Round 3: Based on the results of round 1 and round 2, it is logically decided which original samples cannot be positive. The remaining original samples are potential 'positives' that must be analysed individually to identify the true 'positives'.

Therefore, the rationale of the procedure rests on discarding original samples, $\mathrm{S_i}$, that cannot be positive according to the test outcomes of the pooled samples, and otherwise conducting individual tests.

Formally, rounds 2 and 3 are defined as follows:

Let D be the set of original samples $\mathrm{S_i}$ $i = 1, \ldots, I$ that will be discarded. For each of the original samples, if the logical value of proposition (5) is 1, then $\mathrm{S_i} \in D$.

$$lv\left(\bigvee_{t=1}^{T}(x_{ti} = 1) \bigwedge (lv(\mathrm{P_t}) = 0)\right) \tag{5}$$

2.4. A case-study

For illustrative purposes, a particular case (*Case* 1 from Fig. 1) in the detection of *Listeria* is considered, whose outcomes y_t, the PCR's of the pooled samples, are showed in Table 2.

As pooled samples 2 and 5 test negative, we can logically discard the original samples $\mathrm{S_1}$, $\mathrm{S_2}$, $\mathrm{S_4}$, $\mathrm{S_6}$, $\mathrm{S_7}$, $\mathrm{S_9}$ and $\mathrm{S_{10}}$, marked with green '1'. The remaining original samples, $\mathrm{S_3}$, $\mathrm{S_5}$ and $\mathrm{S_8}$, in red bold, cannot be discarded so they are potential positive samples that must be tested individually.

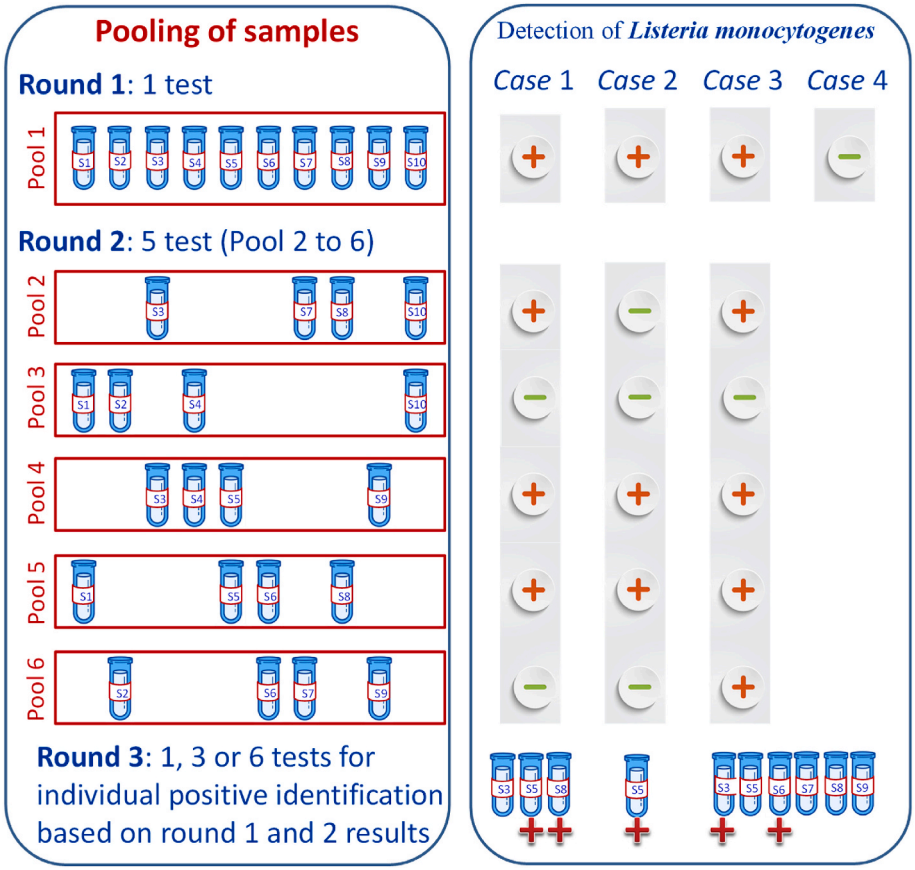

Fig. 1. Outline of the rounds carried out in the Detection of *Listeria monocytogenes* by means of PCR and logical solution for the identification of positive samples.

Table 2
Design matrix for the pooling and test outcomes y_t of the pooled samples (negative/positive) in *Case* 1 from Fig. 1. '1' represents the original samples included in each of the 6 pooled samples. Original samples (S_i) marked with green '1' are logically discarded in each pool. Original samples (S_i) not discarded in any pool (in red bold) have to be tested individually.

Pooled samples	Original samples										Outcome	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	Positive	1
P_2	1	1	0	1	0	0	0	0	0	1	Negative	0
P_3	0	0	1	1	1	0	0	0	1	0	Positive	1
P_4	1	0	0	0	1	1	0	1	0	0	Positive	1
P_5	0	1	0	0	0	1	1	0	1	0	Negative	0
P_6	1	1	1	1	1	1	1	1	1	1	Positive	1

3. Software and experimental

3.1. Software

The procedure for identifying the original samples to be analysed, explained in Section 2.3, is carried out by a function, *solver1*, written in MATLAB [54] and showed in Annex A of supplementary data, has been used.

3.2. Experimental procedure

The procedure of construction of the pooled samples and their analysis has been applied to the detection by polymerase chain reaction (PCR) of the pathogen *Listeria monocytogenes* and the allergen *Pistachio*. The *Listeria monocytogenes* is regulated by Commission Regulation (EC) No 2073/2005 of November 15, 2005 on microbiological criteria for foodstuffs, whereas the presence of *Pistachio* by Regulation (EU) No 1169/2011 of the European Parliament and of the Council of October 25, 2011 on food information provided to consumers.

The PCR analyses have been carried out by AGROLAB IBERICA, S.L. U. Burgos by accredited method (Standard UNE-EN ISO/IEC 17025 accreditation nº 258/LE2068).

3.3. Preparation of samples

Two types of food matrices have been considered: rice flour and liquid sample (with carbohydrates as the main component). With each of them, four batches have been prepared, each with 10 samples. In each case with the rice flour matrix, one, two or no samples have been doped with the *Pistachio* allergen. Similarly, in liquid samples with carbohydrates as the main component: one, two or no samples have been doped with *Listeria monocytogenes*.

The doped samples have been prepared with a double-blind protocol. According to the suggested methodology, the samples to be analysed individually are determined after getting the outcomes of round 2, so neither the authors of this work nor the analysts of AGROLAB could know which samples had been doped. Therefore, this task was performed by a third person, independent of both analysts and researchers.

The protocol for doping the samples has been the same with *Listeria monocytogenes* and *Pistachio*. Four batches of 10 samples were prepared with their corresponding matrices. In the first batch, one sample was randomly chosen to be doped. In the second batch, two samples, randomly chosen out of the 30 possibilities involving the analysis of 3 individual samples in round 3, were doped. Likewise, in the third batch, two samples were doped, but they were randomly chosen from the 15 possibilities involving the analysis of 6 individual samples in round 3. Finally, none of the 10 samples in the fourth batch was doped. The four batches were sent in random order to the laboratory, which did not know the number and position in the batch of the doped samples. The laboratory made two aliquots of each individual sample, using one of them to prepare the pooled samples of each batch according to Table 1, and then delivered the outcomes.

Once the results were analysing with the proposed methodology, the laboratory was tasked with analyzing the individual samples of round 3 in each batch, and finally the corresponding results of these new analyses were received.

4. Results and discussion

4.1. Efficiency and accuracy of the proposed pooling method

The comparison of the suggested pooling procedure with other pooling algorithms has been conducted using a prevalence of 0.05, and several combinations of sensitivity, S_e, and specificity, S_p, of the analytical test, with values of 0.90, 0.95, 0.99 and 1.00 (see Tables 3 and 4). Considering all the groups of size 10 from a population of 100,000 individual samples, the application of the chemical analysis has been reproduced computationally for each combination of S_e and S_p different from 1.00. This means, first, generating 100,000 numbers (zeros or ones) which represent the status of individual samples (negative or positive), where the probability of '1' (being positive) equals the prevalence. Secondly, these samples are divided into 10,000 initial groups of size $I = 10$, and for each of them, the design matrix, detailed in section 2.1, is used to make the pooled samples (P_1 to P_6). Thirdly, the true outcome of every pooled sample is logically determined: the logical value will be '1' (positive) if at least one of the individual samples is positive, and '0' (negative) if all of them are negative. Finally, from the true logical values of the pooled samples, using the logical procedure described in section 2.2 (round 1 to 3), the true logical values for the individual samples, $lv_{true}(S_i)$ are found.

It must be noted that every time the analytical test is applied to a sample, whether individual or pooled, a logical value not necessarily the true one is obtained due to the particular combination of S_e and S_p of this analytical test. So, once the whole procedure has finished, logical values are obtained for each individual sample, $lv_{obt}(S_i)$.

If $lv_{obt}(S_i) = lv_{true}(S_i)$ the suggested procedure performs a correct classification. Otherwise, the classification of sample S_i is wrong. From these results, some figures of merit have been obtained, particularly, the efficiency and the accuracy of the pooling strategy.

4.1.1. Expected number of tests

The assessment of the group testing proposal is conducted in terms of

Table 3
Comparison of pooling algorithms. Optimal Test Configuration (OTC) and expected number of tests per individual sample E(T)/I, according to the sensitivity and specificity of the analytical test used in the screening method.

Pooling algorithm	OTC	Sensitivity/Specificity					
		0.99/0.99	0.95/0.95	0.90/0.90	0.99/0.90	0.90/0.99	1.00/1.00
Supersaturated-based design		0.366	0.370	0.374	0.416	0.332	0.370
2-S Hierarchical	10,1	0.503	0.511	0.521	0.557	0.467	0.510
3-S Hierarchical	4,3,3	0.380	0.381	0.383	0.424	0.342	0.380
2D array 3x3 w/master pool	3x3	0.699	0.719	0.730	0.796	0.643	0.622
2D array 3x3 w/o master pool	3x3	0.741	0.794	0.849	0.843	0.749	0.653
2D array 4x4 w/master pool	4x4	0.565	0.580	0.585	0.639	0.520	0.895
2D array 4x4 w/o master pool	4x4	0.581	0.621	0.661	0.658	0.588	0.911

Table 4
Comparison of accuracy for pooling algorithms. Optimal Test Configuration (OTC), pooling sensitivity, PS_e, and pooling specificity, PS_p, depending on the sensitivity and specificity of the analytical test used in the screening method.

Pooling algorithm	Sensitivity/Specificity									
	0.99/0.99		0.95/0.95		0.90/0.90		0.99/0.90		0.90/0.99	
	PS_e	PS_p	PS_e	PS_p	PS_e	PS_p	PS_e	PS_p	PS_e	PS_p
Supersaturated-based design	0.9665	0.9999	0.8351	0.9953	0.7241	0.9897	0.9697	0.9872	0.7022	0.9994
Pooling size	10		10		10		10		10	
2-S Hierarchical	0.9801	0.9981	0.9025	0.9082	0.8100	0.9719	0.9801	0.9735	0.8100	0.9982
OTC	5,1		5,1		6,1		5,1		5,1	
3-S Hierarchical	0.9703	0.9990	0.8574	0.9930	0.7290	0.9853	0.9703	0.9874	0.7290	0.9988
OTC	9,3,1		9,3,1		12,4,1		9,3,1		12,4,1	
2D array w/o master pool	0.9705	0.9905	0.8581	0.9926	0.7302	0.9842	0.9705	0.9839	0.7301	0.9986
OTC	10,1		10,1		10,1		9,1		11,1	
2D array w/master pool	0.9986	0.9736	0.9930	0.8600	0.9858	0.7091	0.9842	0.7617	0.9988	0.9660
OTC	100,10,1		100,10,1		100,10,1		81,9,1		121,11,1	

efficiency, particularly, the expected number of tests per individual (original) sample that is necessary to identify all the positives, E(T)/I. Results are showed in Table 3.

Last column of Table 3 shows E(T)/I when the sensitivity and specificity of the analytical test are not taken into account, i.e., with S_e=S_p = 1.00. In this case, the algorithm presented here, which is not strictly hierarchical, results in an expected value per individual E(T)/I = 0.37, using Eq. B1 in Annex B of Supplementary data. That is, to decide on each original sample whether it is positive or not, it is only expected to perform 0.37 analytical tests on average. This means a reduction of 63% compared to individual tests.

Comparisons of this efficiency with those of alternative pooling algorithms are shown in Table 3. For a two-stage (2-S) hierarchical method, the Optimal Test Configuration (OTC) with a pooled sample of size 10 in the first stage and samples of size 1 in the second stage, results in efficiency equal to 0.51 (49% of reduction). The three-stage (3-S) hierarchical method, where the OTC has an initial pool size of 10, subsequent pool sizes of 4, 3, and 3 in the second stage and individual samples in the third stage, performs better: E(T)/I = 0.38, reducing the expected number of tests by 62% when compared to individual testing.

2D-arrays have been used with and without master pool, both with a 3x3 grid and a 4x4 grid. When the master pool is included, all the original samples in the array are amalgamated for the first stage of the algorithm. If the master pool tests positively, rows and columns (of size 3 or 4) are tested in the second stage of the algorithm. Individual testing needs to be performed at the intersection of positive rows and columns. Also, whenever a row (column) tests positively without any column (row) testing positively, all samples within the positive row (column) need to be tested individually. A 3x3 array with master pool (which loses one of our 10 original samples) gives an efficiency of 0.69, and thus a reduction of 31%. Likewise, a 4x4 array with master pool (which would require six additional original samples) leads to an expected number of tests of 8.95 and a corresponding E(T)/I of 0.56 (8.95/16), which means a reduction of 44%. Without using a master pool, both procedures reach worse results: efficiency reduces by 27% and 43%, respectively.

When S_e and S_p do not equal 1, computation of E(T)/I for the pooling procedure suggested is performed by the 10,000 groups of size 10, detailed above. For the remaining pooling procedures in Table 3, the computation has been carried out by means of an app in Ref. [55] which implements the algorithms developed in Ref. [48]. In hierarchical algorithms, the optimal test configurations (OTC) for an initial pool size of I = 10 have been used. In 2D array algorithms, the two closest initial sizes to 10, which are 9 or 16, have been considered. The expected number of tests per individual sample is showed in columns 3 to 7 of Table 3.

Clearly the best values are always seen for the proposed pooling algorithm (supersaturated-based design), the closest being the optimal 3-S hierarchical of size 10. Therefore, the suggested procedure, even if it has not been optimized, shows higher efficiency than other methods.

4.1.2. Accuracy measures

In addition to the expected number of tests, the accuracy of correctly classifying truly positive and negative individuals is also important. As defined in section 2.2, the outcome of testing the i-th (i = 1, …, I) sample is $lv_{ob}(S_i) = 1$ if it is diagnosed positive, and $lv_{ob}(S_i) = 0$ if it is diagnosed negative. Likewise, $lv_{true}(S_i) = 1$ or $lv_{true}(S_i) = 0$ are used to denote the true status of the sample. The probability of a correct positive diagnosis after applying the pooling procedure to the i-th sample is named 'pooling sensitivity', defined as $PS_e = p(lv_{ob}(S_i) = 1 | lv_{true}(S_i) = 1)$. Similarly, the 'pooling specificity', $PS_p = p(lv_{ob}(S_i) = 0 | lv_{true}(S_i) = 0)$, is the probability of a correct negative diagnosis.

Table 4 shows the values of PS_e and PS_p for different pairs of sensitivity/specificity of the analytical test used in the screening and a prevalence p = 0.05. The results for the algorithm presented here have been obtained from 100,000 simulated cases, while those of the remaining algorithms have been taken from Table S2 of the Supporting Information of reference [45], where PS_e and PS_p refer to the optimal configuration of the respective pooling algorithm. For the optimization of the hierarchical algorithms, a master pool ranging from 3 to 40 was considered, while for 2D arrays, the same range of group sizes (rows and

columns) was taken. For example, in the 3S Hierarchical, an OTC 12,4,1 means an initial pool of 12 samples in the first stage, pooled samples of size 4 in the second stage and samples of size 1 in the third stage. Regarding the OTC for a 2D array w/master pool algorithm, written as 121,11,1 (last row and column of Table 4) means an array of 121 samples, pooled samples of size 11 corresponding to rows and columns, which finally leads to test individual samples (size 1). For the proposed algorithm, supersaturated-based design, the initial sample size is 10, and neither the design matrix structure nor the pooled sample size is optimized.

In pooling algorithms 2 to 5 of Table 4, PS_e and PS_p depend on the OTC selected, which in turn, depends on the S_e y S_p considered for the analytical test.

For any pair sensitivity/specificity, our proposal shows higher PS_p compared to the remaining algorithms. PS_e is a bit lower, particularly when compared to the 2D array w/master pool algorithm, which uses a big master pool (100 samples). However, it should be noted that 2D array algorithms have a much lower efficiency (see Table 3). On the other hand, there is a great similarity between the results of the proposed algorithm and those of the 3-S Hierarchical for the optimal configuration.

The proposed algorithm shares with the others (except the 2D array with master pool of 121 samples), the asymmetric effect of S_e and S_p on PS_e and PS_p. When S_e is low, there is a remarkable decrease in PS_e, while this does not occur when S_p is low, since PS_p does not experience that drop (with the exception mentioned). This can best be seen in the last four columns of Table 4, which correspond to the sensitivity/specificity pairs 0.99/0.90 and 0.90/0.99.

Another way to assess the accuracy of a pooling algorithm is through the expected number of hits, E(C), which for the proposed algorithm ranges from 9.916 to 9.990 for an initial master pool of size 10.

4.2. Pathogen *Listeria monocytogenes* and allergen Pistachio testing

The results of the detection of the pathogen *Listeria monocytogenes* are represented in Fig. 1. The left panel shows the pooling procedure suggested. It should be noted that, according to the description above, the 'master pool' is tested in the first round (Pool1), although in the design matrix, this corresponds to the sixth row.

The right panel shows the PCR outcomes of the pooled samples: positive (bold plus symbol) or negative (green minus symbol).

Regarding the detection of *Listeria*, *Case* 3 results in only one negative outcome (the second pooled sample or Pool 2, thus discarding original samples 1, 2, 4 and 10, so the six remaining original samples must be individually analysed (round 3). However, in *Case* 2, three outcomes are negative (Pool 1, 2 and 5) so almost all the original samples can be ruled out and just original sample 5 has to be retested. As for *Case* 1, which corresponds to the outcomes showed in Table 2, two pooled samples (Pool 2 and 5) test negative, and therefore original samples 3, 5 and 8 must be tested individually. Naturally, when the 'master pool' tests negative, as in *Case* 4, no further test is required.

Anyway, original samples in round 3 are potentially positive but individual testing shows not all of them are actually positive: a maximum of 2 are detected in each case, reflecting our experimental conditions. The true positive samples were S_5 and S_8 in *Case* 1, S_5 in *Case* 2, and S_3 and S_6 in *Case* 3.

Similarly, the results of the detection of the allergen *Pistachio* can be seen in Fig. 2. The left panel is the same as in Fig. 1, identical design matrix, but the right panel shows a different set of PCR outcomes of the pooled samples. As explained above, no additional test is required when the 'master pool' is negative (*Case* 3), but otherwise, the number of original samples which are tested individually in round 3 (sample S_9 in *Case* 1, samples S_1, S_4 and S_5 in *Case* 2 and samples S_1, S_2, S_6, S_7, S_8 and S_{10} in *Case* 4) increases as the number of pooled samples that test

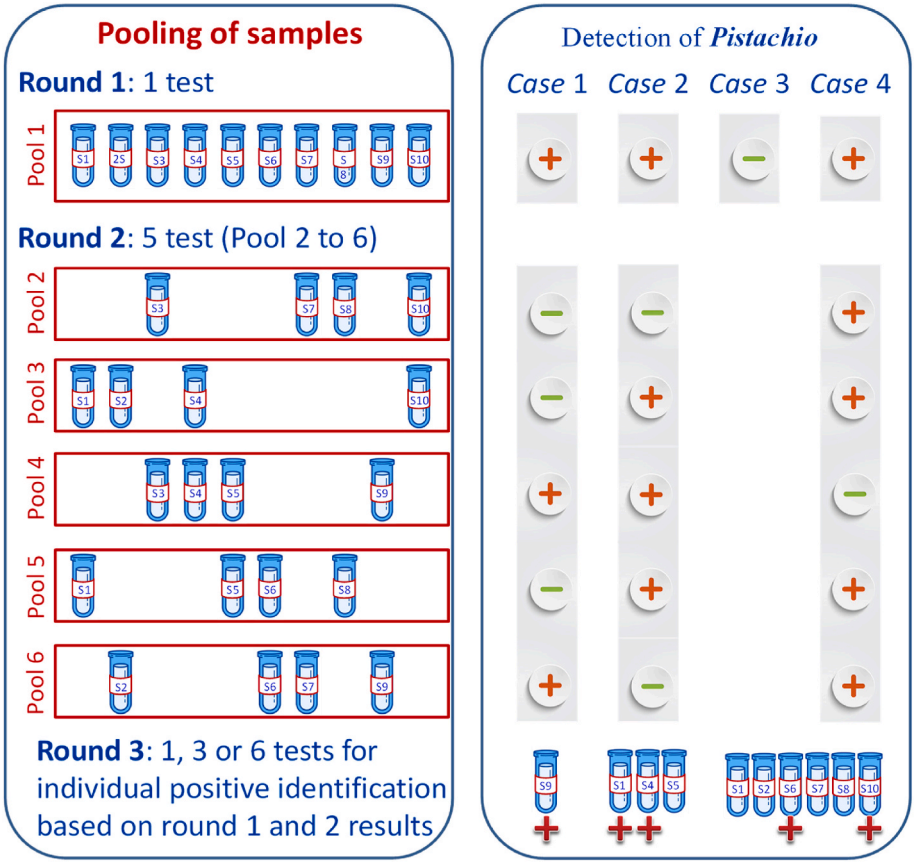

Fig. 2. Outline of the rounds carried out in the Detection of *Pistachio* by means of PCR and logical solution for the identification of positive samples.

negative decreases (three in *Case* 1, two in *Case* 2 and one in *Case* 4). The actual positive samples were S_9 in *Case* 1, S_1 and S_4 in *Case* 2, and S_6 and S_{10} in *Case* 3.

In summary, all samples doped with either *Listeria* or *Pistachio* in our experiment, positive samples, have been correctly identified with the logical methodology developed in section 2.

5. Conclusions

The described design matrix, based on a half-fraction of a Placket-Burman design, along with the modeling of the problem as a logical system of equations, solved through the suggested logical procedure, proves high efficiency compared to other sample pooling strategies and algorithms.

For a prevalence of 0.05 and 10 original samples, the expected number of samples to be analysed for each original sample is 0.37. This means reducing the expected number of tests by 63% compared to individual testing. In addition, the accuracy of the algorithm proposed is better or similar, depending on the sensitivity and specificity of the analytical test, to that of other published algorithms, with an expected number of hits ranging from 99.16 to 99.90%.

The procedure can be implemented for routine analysis because the pooling structure (design matrix) is fixed and the analysis of the results of round 2 can be performed online.

This work opens a line of research that will continue with the optimization of the design matrix, its dimension and structure.

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

Data and code are included in the manuscript

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Appendix A. Supplementary data

Supplementary data to this article (data and code) can be found online at https://doi.org/10.1016/j.chemolab.2023.104902.

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