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Chemometric strategies assessment for the quantitation of dyes using fluorescence spectroscopy

Avaluació d'estratègies quimiomètriques per a la quantificació de pigments mitjançant espectroscòpia de fluorescència

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“Nothing in life is to be feared; it is only to be understood”

Marie Curie

Agrair en primer lloc als meus tutors, en Joaquim i l’Anna. Moltes gràcies per la vostra ajuda en tot aquest temps i per tot el que he après.

Agrair també a la meva família, per la seva paciència i pel seu suport en tot aquest temps.

Finalment, agrair als meus amics, perquè en tot aquest temps no he estat sola i perquè m’he pogut recolzar en ells.

REPORT

IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDG)

One of the main objectives of this project is to quantify food dyes which, as further discussed, may have a negative impact on human health. Thus, this project can be aimed to fulfil the first **P**, which corresponds to **People**. Within the different goals involving this group, the specific goal that this project could fit in would be "SDG 3: Good health and well-being". That is because some food colourings are banned in some countries because of their toxicity and their impact on human health, therefore, their identification or quantification helps to guarantee a good health and well-being to humankind.

In addition, this project aims to quantify food dyes using fluorescence spectroscopy, which is a technique not widely used for the quantitation of food dyes. This technique has its advantages and, if the quantitation of food dyes through it is successful, it could be innovative. For this reason, this project could fit to fulfil the third **P**, which is **Prosperity**. Between the different goals in this group, the specific goal that could be achieved among this project would be "SDG 9: Industry, Innovation and Infrastructure".

Furthermore, this project was carried out on the installations of IDAEA-CSIC and regarding to CSIC, as a research institution, their research involves multiple fields. For this reason, their different projects cover almost all the sustainable goals. In the specific case of IDAEA (Institute of Environmental Assessment and Water Research) its main objectives are focused on the environment, thus, their goals are focused on achieving the second and the third **P**, which are **Planet** and **Prosperity**. For this reason, the goals that will be achieved along this project are aligned with the goals that the institution has set to fulfil.

CONTENTS

1. SUMMARY

Food colourings are additives very common nowadays in the food industry. Its main applications are to give a visual value to the food and to give the costumer an idea about the quality of it. Furthermore, other uses are to recover the colour that some products may lose during the commercialization due to light exposure, temperature changes and humidity. Moreover, food colourings are also used to enhance the natural colours of the food and its appearance or to give an idea about its condition.

Even though its advantages, use of food dyes is very restrictive for human consumption. An excessive intake of food colourings may lead to a variety of health issues. Nowadays, those diseases are mainly related to allergies or hyperactivity. For this reason, the use of food dyes must be regulated, and their quantities in edible foods must be controlled.

There are plenty of ways to analytically quantify food dyes. Fluorescence spectroscopy is an option to quantify food colourings which has benefits such as its high selectivity and sensibility.

The main objective of this project is to take advantage of fluorescence properties to analyse mixtures of different food colourings with different complexity. Afterwards, different multivariate analysis strategies will be tested with the fluorescence data obtained to build models able to quantify different food colorants.

Therefore, different chemometric models based on MCR-ALS were built considering the differences between the different fluorescence acquisition modes (single or multiple excitation wavelengths). As a result, the models built with fluorescence emission at a single wavelength using MCR-ALS and calibration constraints seemed to explain the data better than the models built with 3D excitation emission spectra using classical MCR-ALS constraints.

Keywords: food colourings, fluorescence spectroscopy, chemometrics, quantitation, Multivariate Curve Resolution

2. RESUM

Els colorants alimentaris són additius alimentaris molt emprats avui dia en la indústria alimentària. S'utilitzen principalment per aportar un valor visual als aliments i determinar la qualitat d'aquests. A més, també són utilitzats per recuperar el color que certs aliments podrien perdre en el procés de comercialització degut a diversos factors externs com poden ser l'exposició a la llum, variacions de temperatura i la humitat. També s'empren per potenciar els colors naturals que poden tenir certs aliments i també són utilitzats per donar una idea de quin és l'estat de l'aliment.

Tot i els beneficis que pugui comportar l'ús dels colorants alimentaris, el seu ús està molt regulat per a la seva ingesta, ja que aquests en excés poden tenir conseqüències en la salut. Avui dia aquests es relacionen amb al·lèrgies i hiperactivitat. Per tant, la seva quantificació i regulació és important.

Actualment, hi ha diverses maneres per quantificar de manera analítica els colorants alimentaris, no obstant, en la seva majoria es poden quantificar mitjançant espectroscòpia de fluorescència, el qual presenta les seves avantatges com per exemple la seva elevada sensibilitat i selectivitat.

L'objectiu principal d'aquest projecte és aprofitar l'elevada sensibilitat d'aquesta tècnica i realitzar l'anàlisi de mescles de diversos colorants en diferents graus de complexitat mitjançant la seva fluorescència. Posteriorment, amb les dades obtingudes, es pretén realitzar una anàlisi d'aquestes mitjançant una aproximació multivariant per establir diferents models que siguin capaços de quantificar els diferents colorants.

Per tant, es van construir diferents models basats en MCR-ALS depenent de si les dades obtingudes eren espectres a una longitud d'ona fixa o variable. Es va observar que la resolució dels primers espectres emprant restriccions de MCR-ALS i de calibratge descrivien millor les dades que els models construïts amb espectres 3D d'excitació-emissió.

Paraules clau: colorants alimentaris, espectroscòpia fluorescència, quimiometria, quantificació, Multivariate Curve Resolution

3. INTRODUCTION

3.1. USE OF DYES IN THE FOOD INDUSTRY

Colour has always been important to the consumer's perception of food. Food colourings are common nowadays and its uses in the food industry are very diverse. For example, they are used to indicate the condition of the food at a particular time and to enhance its natural appearance. Food colourings can be added to compensate for colour losses during the manufacturing process caused by various processes such as exposure to light, temperature fluctuations and humidity (1). By defining the main purposes of colorants, they can be considered food additives, as defined by the WHO, "substances added to food to maintain or improve its safety, freshness, taste, texture or appearance"(2).

3.1.1. Natural and synthetic food dyes

Sources of colorants are wide, but food dyes are mainly classified as natural and synthetic, according to their origin.

3.1.1.1. Natural food dyes

Natural food colourings can be found mainly in plants. These type of food dyes are not widely used because they tend to be unstable due to pH and temperature changes. Additionally, they tend to oxidize, which is an inconvenient because colour changes may take place. Moreover, natural colorants tend to be less pigmented than synthetic food colourings, which makes the manufacturing costs higher (3). For these reasons, synthetic dyes are primarily used in the food industry.

Some examples of natural colorants are carotenes (E160a), anthocyanins (E163), annatto (E160b), betanin (E162) and chlorophyllin (E140).

3.1.1.2. Synthetic food dyes

Synthetic food colours are mainly obtained by chemical reactions and are mainly used in the food industry for the reasons mentioned in the previous section. These types of dyes are usually

more pigmented, so not as many compounds need to be used and manufacturing costs are lower. Also, unlike natural food colourants, synthetic dyes have a longer shelf life due to their high stability.

Among the synthetic dyes used in the food industry, azo dyes are the most common on the market. These dyes consist of organic molecules containing an azo group (R-N=N-R'), where R and R' can be an alkyl or an aryl group.

Some examples of azo dyes are Tartrazine (E102), Carmoisine (E122) and Allura Red AC (E129) (Figure 1).

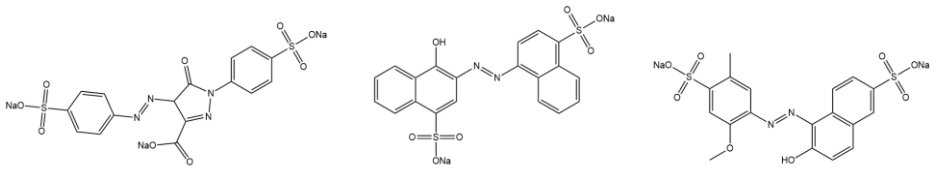


Figure 1: Structure of some azo dyes

3.1.2. Health concerns and EU regulations on the use of food dyes

The use of food colorants in the food industry is very common and important nowadays. However, their use is not without controversy, as they can be toxic and can harm human health if ingested in large quantities (3). For this reason, regulations have been enacted to control the amounts of food dyes in foods, including a ban on many dyes in certain countries. On the other hand, there is also a problem with the food dyes that are allowed. This is because the use of food dyes has recently been linked to allergies and hyperactivity in children (1,3–6).

These regulations attempt to control the amounts of food colourings in food. In the European Union, food additives can be classified in 26 main groups depending on their function on food. The most recent regulation, Regulation (EC) No 1333/2008, establishes the conditions where food colourings should be used. It is said that colorants can only be used in these 3 cases:

1. Compensate any colour loss due to manufacturing processes.
2. Enhance the appearance of foods.
3. Give colour to foods that are colourless.

Nowadays there are 40 colorants permitted by the EU, where 15 of these colorants are synthetic dyes (1).

Labelling of food dyes is also regulated by the European Union. Every food additive is identified with an E number. Food colourings are characterised by having E1xx numbers. Moreover, in the legislation mentioned, in Annex V mentions that certain food colourings should include information regarding the possibility of causing particular diseases.

3.1.3. Instrumental techniques to quantify food dyes

Since food dyes are regulated, it is important to quantify the amounts of dyes in food and to determine whether the dyes used on different foods are banned or not. Nowadays there are plenty of ways to quantify food dyes. Since food dyes present colour, they can be measured by UV-Vis spectroscopy and calculate their concentration with Lambert-Beer's law. However, optic methods need to be joined to separation techniques to quantify food dyes. Additionally, there are some HPLC methods that can be used to analyse up to 20 dyes simultaneously on food samples (7). Some HPLC methods can be coupled to mass spectroscopy too since each food dye has different molecular weight. In Table 1, the techniques mentioned are compiled, with their advantages and inconveniences.

Table 1: Instrumental techniques to quantify food dyes

Technique	*Advantages*	*Inconveniences*	*References*
UV-Vis spectroscopy	-Appliable to all food dyes. -Rapid method.	-Lower sensitivity. -Lower selectivity.	(8–10)
Fluorescence spectroscopy	-High sensitivity. -High selectivity. -Rapid method.	-Not appliable to all food colourings.	(11,12)
HPLC-DAD	-Simultaneous measurements of diverse food dyes. -Obtention of structural data	-Low sensitivity	(13–15)

HPLC-MS	-Simultaneous measurements of diverse food dyes. -High sensitivity -Obtention of structural data	-High-cost instrumentation	(16,17)

However, there is no standard method to quantify food dyes nowadays in the food industry.

3.2. FLUORESCENCE SPECTROSCOPY

Luminescence is the emission of light in a substance when it is in an electronically excited state. It is also characterized for the absorption of light to excite the electrons and the emission of light when the electrons go back from an excited state. Two types of luminescence are described depending on the nature of the excited state: fluorescence and phosphorescence (18). Since in this project, works with fluorescence spectroscopy have been done, in this section, fluorescence fundamentals will be discussed and its applications with food colourings.

3.2.1. Fundamentals

Fluorescence is characterized by the process where an electron that is in an excited state, specifically a singlet state (S_1), goes back to the fundamental state. Basically, the process observed is $S_1 \rightarrow S_0$, mainly. Molecules that undergo this process are called fluorophores. This process can be easily observed through Jablonski diagram. An example of Jablonski diagram is Figure 2, describing the processes observed during fluorescence emission. Other processes can be observed through a Jablonski diagram but the main process that occurs during fluorescence is the one described.

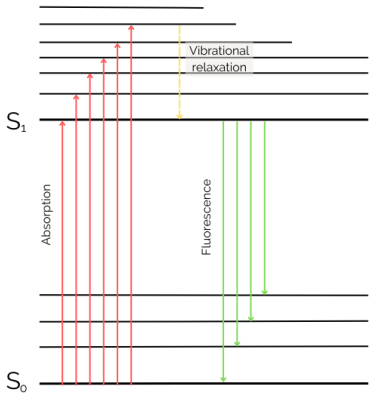


Figure 2: Example of Jablonski diagram

Fluorophores are usually aromatic molecules because π→π* transitions have lower energy, which favour the fluorescence process. Substituents on these molecules influence their fluorescence. Another effect that favours the fluorescence phenomenon is the number of rings that the molecule has: if there are more rings, the effect of fluorescence is favoured. Moreover, if the structure of the molecule is more rigid, the effect of fluorescence is more favourable.

In fluorescence spectroscopy, both excitation spectra and emission spectra can be obtained. However, the most common way to obtain fluorescence spectral data is by the emission spectra.

Some characteristics of fluorescence emission can be described, that are applicable to all fluorophores. On one hand, it is observed that the energy of emission is lower than the energy of excitation. Thus, emission wavelengths are larger than excitation wavelengths. This phenomenon is known as Stokes shift. Furthermore, another characteristic of fluorescence is that the same emission spectrum can be observed at different values of excitation wavelength. This is known as Kasha's rule. Obviously, since it is a scientific observation there will always be exceptions, even though they happen to be less common (18).

The use of fluorescence spectroscopy has its advantages, one of them being its high sensitivity. Additionally, this technique can also be considered a highly specific technique since not all molecules have fluorescence emission. However, if some species don't present fluorescence emission per se, they can present fluorescence signal by derivatizing those compounds with species that present fluorescence emission signal. For these reasons, the use of fluorescence spectroscopy is very common nowadays and its applications are very wide.

3.2.2. Application of fluorescence spectroscopy to food dyes

Since fluorescence presents a high sensitivity, its uses in the food industry, especially in the quantitation of food dyes, can be considered. Most of the food dyes used in the food industry can be considered fluorophores, since most of them are aromatic molecules. However, most of them have an azo group and it can diminish the effect of fluorescence. Despite this drawback, some food colourings present fluorescence signal and their quantification through fluorescence spectroscopy may be an advantage since this technique has a high sensitivity and presents a high selectivity, since not every compound presents fluorescence emission, which eases the quantitation of food dyes in complicated food samples.

Measurements can be made in a quartz cuvette or a well-plate. In a well-plate, the source of light is at a 90º from the sample and a wavelength of excitation is selected. Then, the light reaches the sample and the fluorescence emission that takes place is detected directly from the well, selecting each emission wavelength (19). Measurements of fluorescence in a well-plate have their advantages and inconveniences. In Table 2 figures the advantages and inconveniences of measuring fluorescence emission in a well-plate and in a quartz cuvette.

Table 2: Comparison between obtaining fluorescence emission in a quartz cuvette and a well-plate

	Advantages	*Inconveniences*
Quartz cuvette	-No absorptive material. -Good emission signal. -Resistant to all solvents.	-Slow procedure if a lot of samples are analysed -More sample necessary for the measurements.
Well-plate	-Rapid measurements. -Less sample used.	-Possible interferences depending on the material. -Not resistant to all solvents.

3.3. CHEMOMETRIC DATA ANALYSIS

3.3.1. Multivariate Curve Resolution (MCR)

Multivariate curve resolution (MCR) is a group of chemometric techniques and is also a generic denomination of techniques that are used to solve mixtures analysis problems(20,21). It

builds an additive bilinear model from a data matrix including mixtures decomposing multivariate data into its pure components, considering that each component that makes up the mixtures has a contribution on the measurements. Its main applications are on spectral data and chromatography.

As an example of a MCR application, a data set **D** is obtained after an experiment containing instrumental responses. The main objective of MCR is to describe the information of **D** considering that **D** is the sum of the contributions of each of the components that makes up the measured mixtures. This model can be compacted in the next expression (equation 1) (20,21):

$$\boldsymbol{D} = \boldsymbol{C} \cdot \boldsymbol{S}^{\boldsymbol{T}} + \boldsymbol{E} \qquad \text{(equation 1)}$$

Where **C** are the concentration profile of each component and $\mathbf{S}^{\mathbf{T}}$ are the related pure spectra. The matrix **E** is the variance that cannot be explained by the model.

This method has the advantage to provide valuable chemical data and it is capable to separate the different components that make up a mixture, moreover, it can separate overlapping signals of the components on the measurements made.

MCR methods can be iterative or non-iterative. Nowadays, most common methods are based on iterative methods, in order to describe **D** through the product of matrices **C** and **S**. Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) is widely used since it iteratively optimises matrices **C** and **S** (20,21). To accomplish this, a selection of the number of components, an initial estimation and constraints should be provided. Initial estimations are the starting point where the optimization begins, and they can be both **C** and **S** matrices. An example of a method for determination of initial estimations is “Pure variable selection”, which is one example used in this project. This kind of initial estimation selects the most different rows and columns on a data matrix similarly to the SIMPLISMA algorithm.

On the other hand, constraints are some restrictions that should be applied, so the data obtained has chemical meaning and is described the way we expect in addition to the pure mathematical solutions. Some examples of constraints, which are used along this project, are:

- Non-negativity: this constraint forces the data obtained to acquire positive values. Negative values can be replaced by zeros or by softer algorithms, such as non-negative least squares or fast non-negative least squares. This constraint is applied to all concentration profiles and some instrumental data.

- Closure: this constraint is applied on the concentration matrix on reaction profiles. It follows the mass-balance condition.
- Equality: this constraint is applied when the concentration or spectra profiles are exactly known.
- Correlation: in this constraint, a calibration model is built with some samples (i.e., 25% of the total samples) and the model built is validated with the remaining samples.

3.3.2. Application of MCR to fluorescence spectroscopy

MCR can be used to analyse fluorescence spectroscopic data. As mentioned above, main applications of MCR are in spectroscopic fields and chromatography. For this reason, this kind of data can be analysed. The matrix **D** obtained on the measurements made can be fluorescence emission spectral data, where the rows are the different samples, and the columns are their respective fluorescence spectra. The matrix **C** obtained will contain the concentration profiles of each component that makes up the sample and the matrix **S** obtained will contain the contribution of each component to the spectral data.

Another form of the matrix **D** would be 3D excitation emission spectra. In this new matrix, there are submatrices according to the samples analysed. The matrix **C** obtained will be the concentrations of each sample at every excitation wavelength and the matrix **S** obtained will contain the pure spectra of each component optimised.

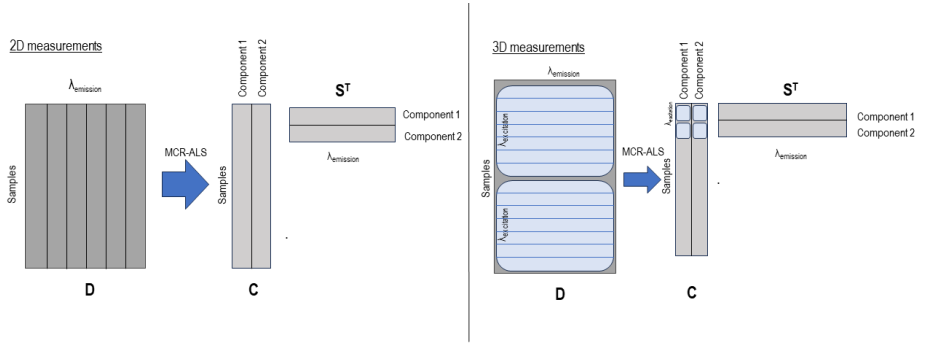


Figure 3: Example on how fluorescence data will be treated. (Left) 2D spectra; (Right) 3D spectra

4. OBJECTIVES

The main objective of this project is to evaluate the analysis through fluorescence spectroscopy different mixtures of food colourings of different complexity, considering binary or ternary mixtures and different degrees of selectivity. The fluorescence data related to the mixtures will be analysed through different chemometric strategies based on MCR-ALS.

To accomplish this main objective, some minor goals must be achieved:

- Determine and optimize the experimental conditions for the analysis of food dyes using fluorescence spectroscopy considering single and multiple excitation wavelengths.
- Test different chemometric strategies based on MCR-ALS to analyse the fluorescence emission data, taking into account the application of various constraints.

5. EXPERIMENTAL SECTION

5.1. MATERIALS AND METHODS

5.1.1. Solvents and reagents

The analysed dyes were from the brand HXDZFX "Food Colouring" (Shenzhen Huahengchuangda Trading Co, China), containing 20 different dyes and only commercial colour names were provided. A preliminary study was made searching information through Scifinder and Reaxys and permitted an initial annotation of each colorant. In Table 3, literature values of maximum absorption in UV-Vis and wavelengths of excitation and emission can be observed. In some cases, the data cannot be found.

Table 3: Initial assignations of food dyes and theoretical values

	Food colouring	*UV-Vis spectroscopy*	*Fluorescence spectroscopy*	
Color		**Max UV spectra (nm)**	**λ_{exc} (nm)**	**λ_{em} (nm)**
Lemon Yellow	Tartrazine	259; 425	310	400
Orange Yellow	Sunset Yellow	480	303	430
Orange	Carotenes	455	-	-
Orange Red	Allura Red AC	504	330	405
Sunset Red	Ponceau 4R	510	555	575
Incense Purple	Amaranth	521; 506	552	576
Red	Carmoisine	520	556	570

Pink	Erythrosine	525	524	574
Rose	Betanine	536	-	-
Cherry Red	-	-	-	-
Navy Blue	Indigotine	610	280	322
Brown	Brown HT	-	-	-
Grape Purple	Delphinidin	500	-	-
Green	-	-	-	-
Yellow Green	Green S	-	-	-
Grass Green	Chlorophylls	663; 431; 459; 646	-	-
Dark Green	-	-	-	-
Blue Green	Patent Blue	635	654	-
Sky Blue	Brilliant Blue	628	-	-
Black	Black PN	596	-	-

Stock solutions were prepared by pipetting 50 µL of each dye and diluting with 2950 µL of water. Since there is no concentration data of the food dyes on the samples, the concentrations given in this project will be volume of stock solution diluted by a certain volume of Milli-Q water.

5.1.2. Equipment

The different instruments used during this project were, an Agilent Cary Eclipse Fluorescence Spectrometer (Agilent Technologies) for fluorescence measurements and Agilent UV-VIS Spectrophotometer 8453 (Agilent Technologies) for molecular absorption UV-Vis measurements.

Other materials used during this project were different well-plates of 96 wells:

- A white well-plate
- A transparent well-plate
- A black well-plate
- A white well-plate with transparent wells

5.2. INDIVIDUAL CHARACTERIZATION OF FOOD COLOURINGS

5.2.1. Fluorescence emission spectra obtained with a quartz cuvette

Measurement solutions were made by pipetting 100 µL and diluting with 3000 µL of water. UV-Vis and fluorescence emission spectra were obtained with a quartz cuvette. In the case of fluorescence spectroscopy, the λ_{exc} was fixed and the fluorescence emission spectra was obtained. In some cases, the qualitative assignation made did not match so 3D spectra were obtained by fixing λ_{exc} and increasing in an interval of 10 nm until 500 nm and obtaining the fluorescence emission at those λ_{exc}.

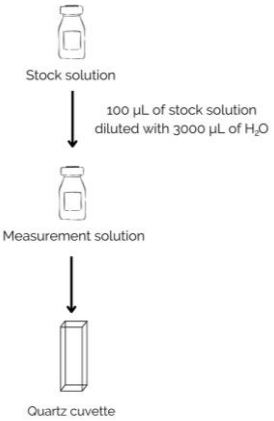


Figure 4: Measurements on a quartz cuvette

5.2.2. Fluorescence emission spectra obtained with a well-plate

After determining which food dyes have fluorescence emission, fluorescence emission spectra were obtained using different well-plates. The food dyes were grouped by having similar λ_{exc} and λ_{em}.

In order to optimize the conditions to obtain fluorescence emission spectra, tests with different volumes, concentrations of food dyes and type of well-plate have been done. To make this optimization, measurements with the food colouring Red (λ_{exc}= 330 nm and λ_{em}= 423 nm) have been done.

From the stock solution, 3 solutions with different concentrations have been made, pipetting different volumes of the stock solution, and diluting with the same amount of water. The most

diluted solution was made from pipetting 100 µL of the stock solution and diluting with 3000 µL of Mili-Q water. The intermediate solution was made pipetting 150 µL of the stock solution and diluting with 3000 µL of Mili-Q water and the most concentrated solution was made by pipetting 200 µL of the stock solution and diluting with 3000 µL of Mili-Q water.

Also, tests with different volumes of solution were done and two different well-plates have been used, a white well-plate and a transparent well-plate. The position of the different solutions and volumes on the different well-plates were the following:

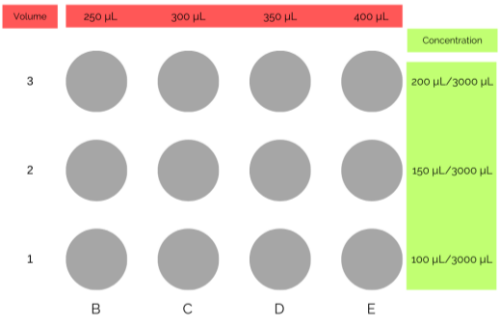


Figure 5: Placement of sample volumes and concentrations on the well-plate

5.3. STUDY OF MIXTURES OF FOOD DYES

A well-plate of 96 wells was filled with binary and ternary mixtures of high selectivity and low selectivity and with different proportions of 3 food dyes: Pink (λ_{exc}= 520 nm and λ_{em}= 550 nm), Cherry Red (λ_{exc}= 510 nm and λ_{em}= 548 nm) and Incense Purple (λ_{exc}= 330 nm and λ_{em}= 425 nm). (See in Appendix 2)

Measurements of fluorescence emission were made using two strategies.

1. Emission spectra were obtained at the excitation wavelength of each food colouring, that is:
    a. An excitation wavelength of 330 nm the emission spectrum was obtained from 350 nm to 500 nm measuring the emission every 1 nm.
    b. An excitation wavelength of 510 nm the emission spectrum was obtained from 530 to 700 nm measuring the emission every 1 nm.

2. 3D spectra were obtained at multiple excitation wavelengths, from 310 nm to 550 nm with an interval of 10 nm, and the emission measured at each excitation wavelength was obtained from 350 to 700 nm measuring the emission every 1 nm.

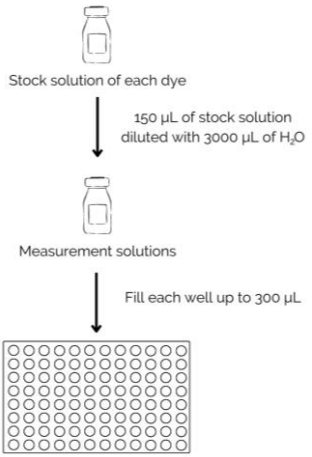


Figure 6: Measurements on a well-plate

5.4. DATA ANALYSIS STRATEGY

The data obtained in the different experiments will be analysed with MCR-ALS applying different constraints. All mixtures were analysed on the same analysis and all the data will be contained in the same matrix. Preprocessing of the data was not necessary. First, data from 2D measurements were joined on the same matrix and analysed testing different constraints for both concentration and spectra matrices. Non-negativity was applied in all the models built. Then, the closure constraint was applied to the concentration matrix. Other models using correlations were built, first considering each food dye individually and then a model was built considering the data from all food dyes simultaneously.

Finally, data from 3D excitation emission spectra was analysed using different constraints for both concentration and spectra matrices. The constraint of non-negativity was applied in all built models. An additional model was built considering the constraint of identification of species on the concentration matrix. However, a previous correction of the matrix was needed since the data contained peaks from scattering, which interfere with the peaks corresponding to the samples.

6. DETERMINATION OF FLUORESCENCE CONDITIONS: DYES AND WELL-PLATES

A comprehensive analysis was conducted on all samples, resulting in the identification of fluorescence emission in 15 out of the 20 food dyes examined. In Table 4 can be observed the experimental values obtained of λ_{exc} and λ_{em} in fluorescence spectroscopy and maximum absorption wavelengths in UV-Vis molecular absorption spectroscopy (values obtained doing measurements on a quartz cuvette).

Table 4: Experimental data of the food dyes

	UV-Vis spectroscopy	*Fluorescence spectroscopy*	
Color	**λ maximum (nm)**	**λ_{exc} (nm)**	**λ_{em} (nm)**
Lemon Yellow	258; 425	310	400
Orange Yellow	241; 254; 443	-	-
Orange	234; 313; 481	280	400
Orange Red	213; 234; 484	330	421
Sunset Red	214; 223; 500	330	420
Incense Purple	216; 322; 521; 629	330	425
Red	215; 329; 505	330	423
Pink	255; 308; 526	520	551
Rose	216; 331; 520	320	422
Cherry Red	216; 525	510	548
Navy Blue	212; 629	320	425
Brown	215; 523; 629	330	420
Grape Purple	256; 410; 629	300	430

Green	256; 307; 409; 629	-	-
Yellow Green	257; 424; 629	-	-
Grass Green	257; 410; 629	-	-
Dark Green	256; 307; 409; 629	290	367
Blue Green	254; 307; 408; 627	275	300
Sky Blue	307; 408; 629	-	-
Black	213; 309; 411; 501	320	430

These values can be compared with Table 3, which includes assignments made for each food dye based on their observed colours, assuming each dye to be a distinct molecule. Such assumptions were necessary due to the absence of information regarding the composition of the samples in the manufacturer's data. It is observed that the assignations made in Table 3 did not totally match the experimental values obtained. This does not suppose a problem, since the main objective of this project is to quantify the dyes, even though the compound is totally identified.

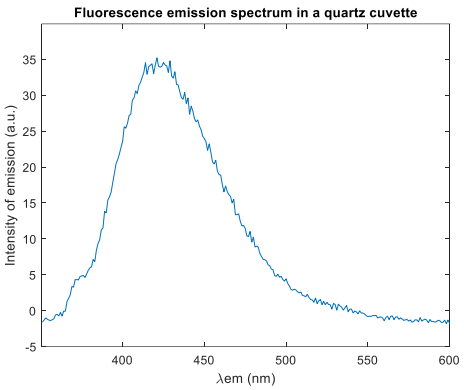
Afterwards, spectra were measured from different well-plates, to identify possible differences between the spectra obtained in a quartz cuvette and a well-plate. Moreover, obtaining the fluorescence emission spectra in a well-plate was necessary since it produces faster results, and it helps the measurement of mixtures of food dyes.

On Figure 7, the fluorescence emission spectra of the food colouring Sunset Red can be observed, measured in a quartz cuvette (a), a white well-plate (b), a black well-plate (c), a transparent well-plate (d) and a white well-plate with transparent wells (e). In the case of the different well-plates, there is some noise that can be due to the plastic well-plates at the region of maximum emission of the food dye. In most of the cases, the signal of the well-plate overlaps with the signal of the food colouring which makes difficult the identification of the peak of the food colouring. In order to identify if the signal comes from the plate 3D excitation emission spectra were obtained.

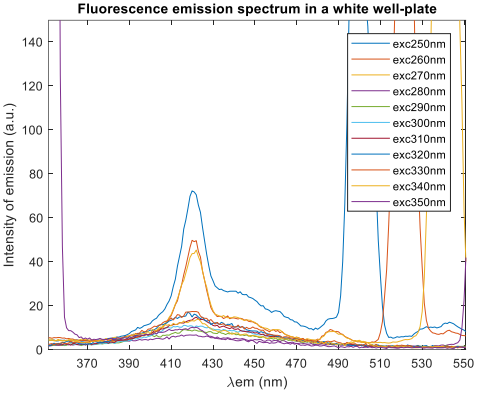
It is observed that the signal observed around 400 nm in each plate is constant at an interval of excitation wavelengths, which means that there is a compound that presents emission fluorescence at that region, which is most likely due to the plastic present on the well-plates or their colour.

It can also be observed that the plates that did not present much noise in the region of maximum emission of the food colouring were the white and black well-plate, but in both cases the peaks of fluorescence emission were a little bit different compared to the spectrum obtained with a quartz cuvette. However, there are a lot of factors that can affect the aspect of the peaks, such as the volume of sample in each plate and type of well-plate used for the measurements, thus, it is important to optimize the conditions for further measurements.

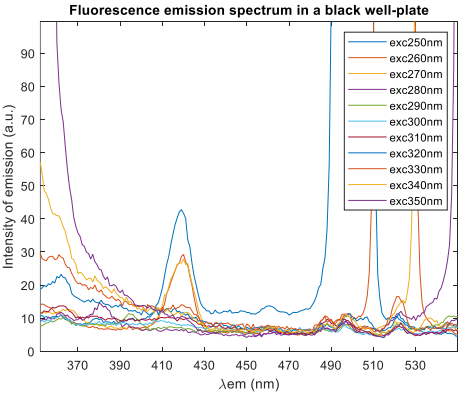
(a)



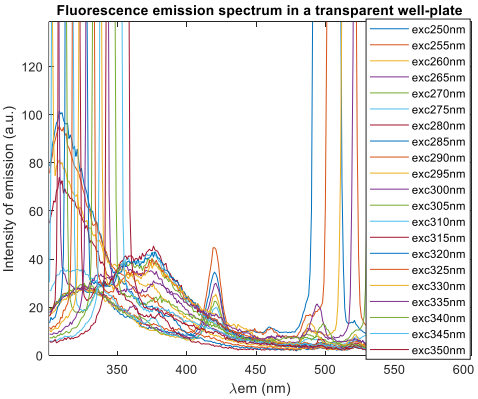
(b)



(c)



(d)



(e)

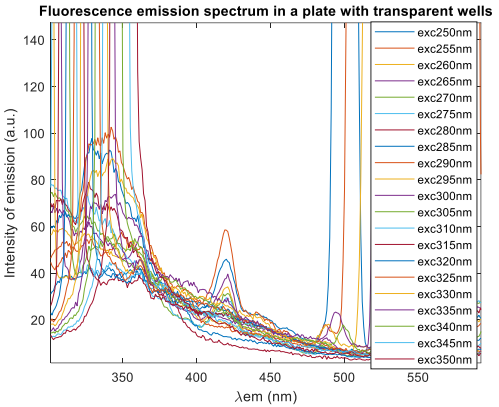


Figure 7: Emission spectra on different sample collectors. (a) Quartz cuvette; (b) White well-plate; (c) Black well-plate; (d) Transparent well-plate; (e) Well-plate with transparent wells

7. OPTIMIZATION ON THE CONDITIONS OF MEASURING IN A WELL-PLATE

Optimization on the conditions of measuring food dyes in a well-plate was necessary, since as observed, some plates presented emission fluorescence in the region of 400 nm where most of the food dyes present fluorescence emission, as seen in Table 4. As it can be seen on Figure 7, there are also differences between the signal of the different well-plates. For this reason, different volumes of food colourings, concentrations of food dyes and different well-plates were tested. All tests in this section were done with the food colouring Red because it is a food colouring that presents high intensity of fluorescence emission in the region of 400 nm, where the well-plates suppose an interference. Measurements at 550 nm were not necessary, since the signal of the samples measured on the well-plates did not differ much from the spectra obtained with a quartz cuvette.

On one hand, in the spectra obtained in the previous section, well-plates were filled halfway with the sample, which is 200 µL because the wells have a capacity of 400 µL. In this case, several volumes of sample were tested, in order to identify which is the ideal volume to measure, starting from 200 µL.

On the other hand, severe concentrations of food dye were also tested, by pipetting different volumes of the stock solution and diluting with the same amount of water. These tests were made with the aim of finding which concentration presents an acceptable signal of fluorescence emission.

The white well-plate and transparent well-plate were tested. Even though the black well-plate presented a favourable signal, measurements with that plate were discarded because the colours in that plate could not be observed. Additionally, this well-plate seemed to be more expensive, which is unfavourable if these well-plates were selected for routine analysis.

In Figure 8 can be observed the spectra obtained at different concentrations and different volumes with an excitation slit of 5 nm in a transparent well-plate.

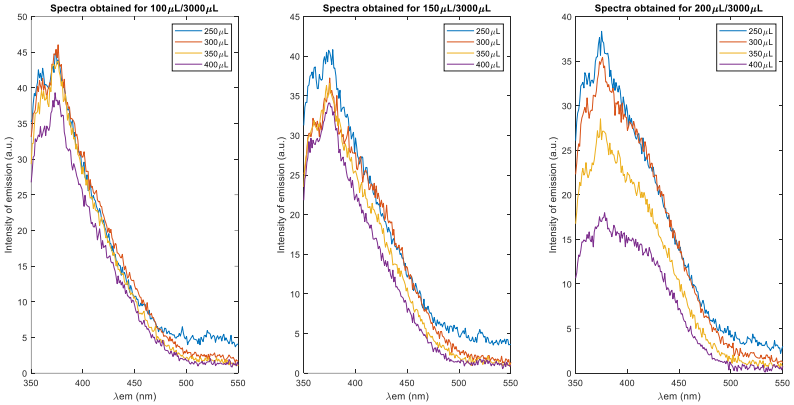


Figure 8: Emission spectra of Red in different concentrations and volumes in a transparent well-plate at an excitation slit of 5 nm. (Left) Most diluted solution; (Middle) Intermediate concentration; (Right) More concentrated solution

However, in Figure 9, can be observed the spectra obtained at different concentrations and different volumes at an excitation slit of 5 nm. Differences between this well-plate and the transparent well-plate were noted.

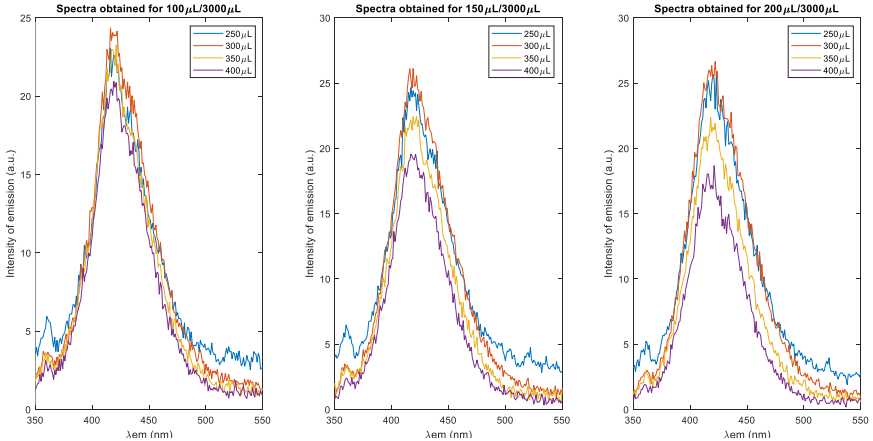


Figure 9: Emission spectra of Red in different concentrations and volumes in a white well-plate at an excitation slit of 5 nm. (Left) Most diluted solution; (Middle) Intermediate concentration; (Right) More concentrated solution

It was observed that the emission peak obtained on the white well-plate was similar to the signal obtained with a quartz cuvette rather than the signal observed on the transparent well-plate.

On the other hand, the tendency with the volume of solution varied. It is noted that in 3 cases, the maximum of emission of fluorescence occurred at a volume of 300 µL of sample. Thus, further measurements of fluorescence were carried out at a volume of 300 µL.

At an excitation slit of 10 nm the signal of fluorescence emission increased, as it can be seen in the scale of the Figure 10 compared to the scale of Figure 9. That is because there was more light passing through the slit, which permitted the signals to be greater.

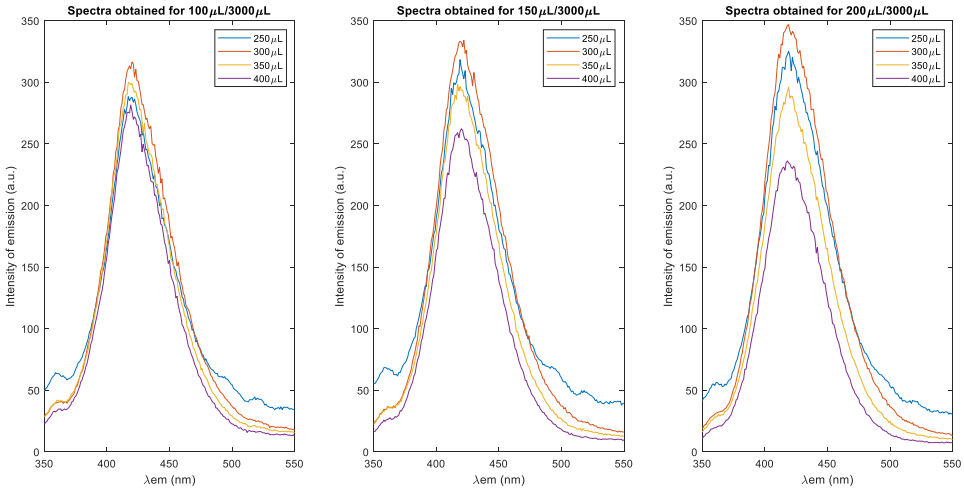


Figure 10: Spectra of Red in different concentrations and volumes in a white well-plate at an excitation slit of 10 nm. (Left) Most diluted solution; (Middle) Intermediate concentration; (Right) Most concentrated solution

Therefore, once the measurements were done, it can be concluded that the well-plate to be used on the next measurements was the white well-plate. The optimum volume of sample that was used is of 300 µL and the concentration of the food dye ideally to be used would be of 150 µL of food dye with 3000 µL of water. The slit used for further measurements would be of 5 nm, because even though the signal with a slit of 10 nm was greater, the signal of the food colouring Pink with a slit of 10 nm surpassed the scale, which was not favourable for further data analysis.

8. CHEMOMETRIC RESOLUTION OF FLUORESCENCE SPECTRA

96 mixtures of different complexity and selectivity were measured through fluorescence spectroscopy with the white well-plate (Appendix 2). The food colourings selected were Incense Purple, Cherry Red and Pink. Those food colourings were selected because Pink and Cherry Red present fluorescence emission at approximately 550 nm, where the mixtures were better resolved. Incense Purple was selected to make mixtures more complex with a food colouring that has fluorescence emission at the region of 400 nm. Another reason why Incense Purple was selected is because it is one of the food colourings that presents a higher emission peak.

8.1. DATA ANALYSIS FOR 2D SPECTRA

Different strategies were applied for the resolution of mixtures. First, data from measurements made at each food dye's λ_{exc} and λ_{em} were joined to the same matrix and the peak at 550 nm was normalised. Figure 11 shows the joined matrix and how appear the signals of the food colourings.

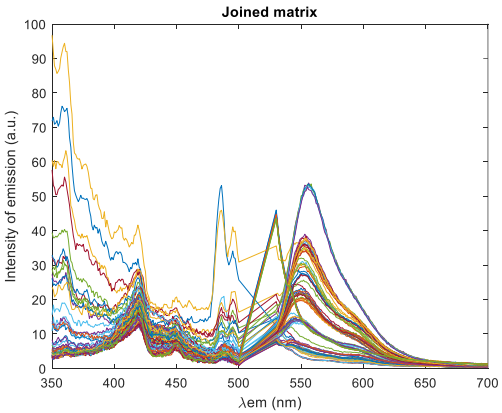


Figure 11: Joined matrix of the data obtained

MCR-ALS was then applied to analyse this data. Four components were necessary, considering a component for each food dye of the mixture and an additional component for the plastic of the well-plate since its signal is not negligible. In all cases in this section, the initial estimation was done manually with a matrix and the containing data were the pure spectra of the 4 components. The first component contained a spectrum of a blank, the second component contained a spectrum of the food colouring Pink, the third component had the spectrum of the food colouring Cherry Red and the fourth component contained the spectrum of the food colouring Incense Purple.

Then, MCR-ALS considering different constraints was tested. Firstly, mixtures were resolved only with the constraint of non-negativity for both matrices **C** and **S**.

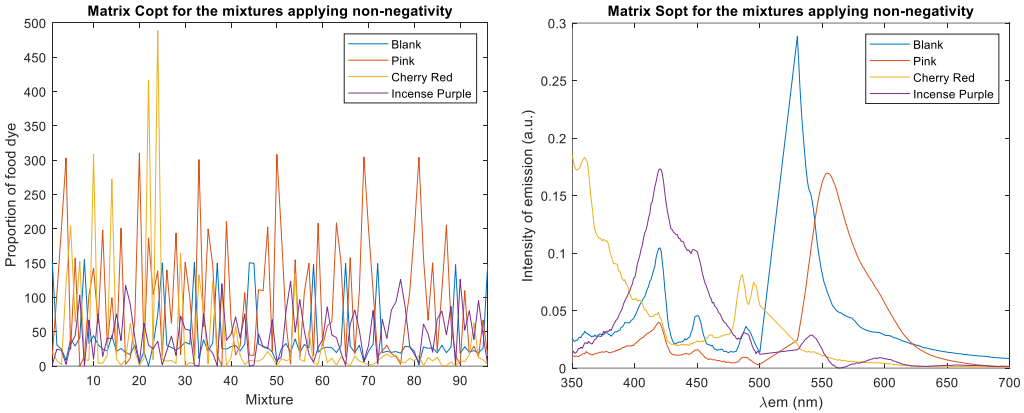


Figure 12: Optimised matrices after applying non-negativity. (Left) Resolved concentrations; (Right) Resolved fluorescence emission spectra

It is observed that the component corresponding to the Blank presents a significant signal, especially on the resolved spectra matrix, and it should not be ignored. That means that the well-plate presents a great contribution. It can also be seen that the concentration profiles are not fully optimized, especially in the case of the food colouring Incense Purple, since its concentration profile does not vary that much amongst the resolved concentrations matrix, which is not true as seen in Figure 13. In the case of Cherry Red, its concentration profile is not fully optimised. It is seen that in the first mixtures the concentration profiles of this food dye are high and on the final mixtures its concentration profiles are very low compared to the theoretical profile. However, in the case of the food colouring Pink, its concentration profile is better optimized since its

concentration profile is close to the theoretical one. Figure 13 shows a comparison between theoretical concentration profiles of each component and the resolved ones. It is seen that Pink is the component that is better resolved, and that the plastic of the well-plate will always have a contribution.

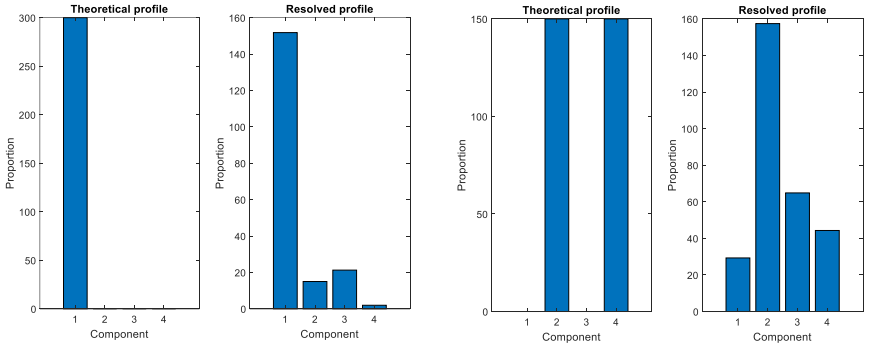


Figure 13: Comparison between theoretical concentration profile and resolved profile. (Left) Blank; (Right) Mixture of Pink and Incense Purple

Since the optimised matrices don't describe the mixtures properly, other constraints are tested. In this case, the constraints of non-negativity and closure have been applied. The constraint of closure was applied to the matrix C assuming that the concentration profiles of all added mixtures sum up to 1. Constraints applied to matrix C were non-negativity and closure and for matrix S non-negativity.

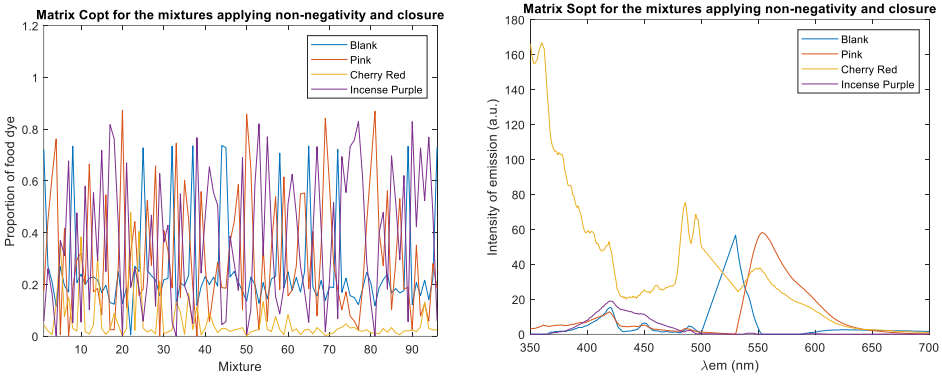


Figure 14: Optimised matrices after applying non-negativity and closure. (Left) Resolved concentrations; (Right) Resolved fluorescence emission spectra

It is observed that in this case the resolution does not improve. The concentration profiles are not properly resolved. In this case, the concentration profile of the food colouring Cherry Red is lower than the concentration profile of the Blank. That is, the plastic of the well-plate presents a great contribution. It is also seen that the concentration profile of the food colouring Incense Purple is better resolved than in the latter case, but it is not a significant improvement. In this case, the food colouring Pink is the food colouring better resolved, as in the previous case.

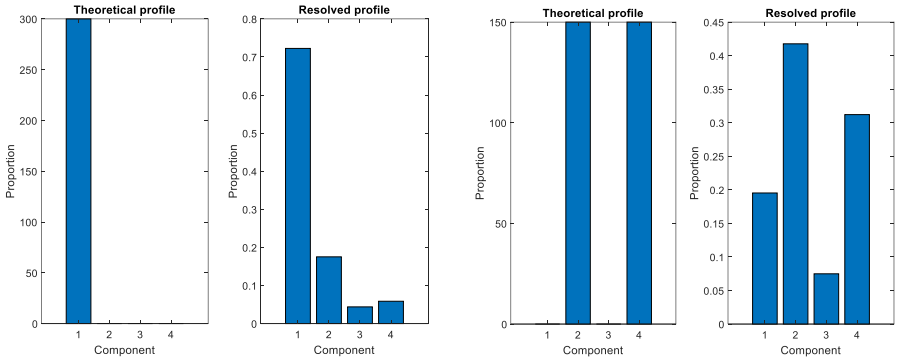
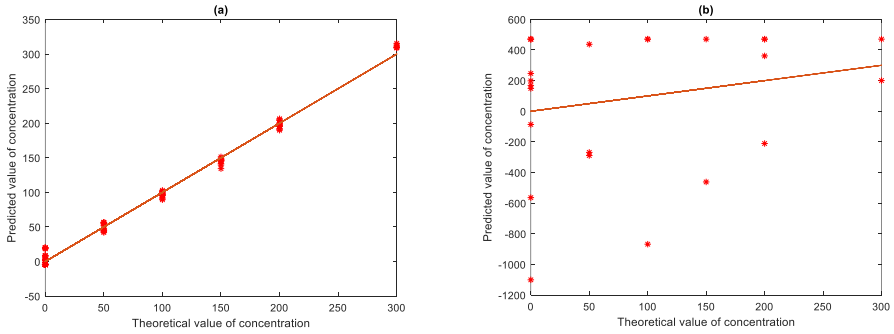


Figure 15: Comparison between theoretical concentration profile and resolved profile after applying non-negativity and closure. (Left) Blank; (Right) Mixture of Pink and Incense Purple

After testing non-negativity and closure constraints and concluding that the resolved concentrations and spectra did not describe properly the mixtures, correlation was used. The first 24 samples were used for calibration and the other mixtures were used as validation samples. Additional to the correlations, the constraint of non-negativity was applied for both **C** and **S** matrices. Firstly, correlations will be tested for each food colouring individually and finally, the correlation of a model with all the food colourings simultaneously were tested.



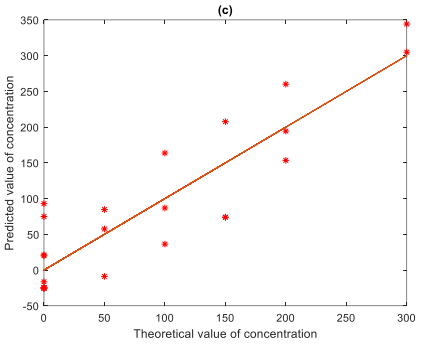


Figure 16: Calibration curves of the food colourings. (a) Pink; (b) Cherry Red; (c) Incense Purple

By observing the Figure 16, the food colouring that is better resolved with MCR-ALS using correlations is the food colouring Pink, because the samples of validation and the samples of calibration form a linear graph. However, this does not apply to the food colouring Cherry Red, because there is a high variability amongst the theoretical values of the concentration and its predicted values. In the case of the food colouring Incense Purple, even though there is some variability, it can be considered that the predicted values of concentration are practically similar to the theoretical ones.

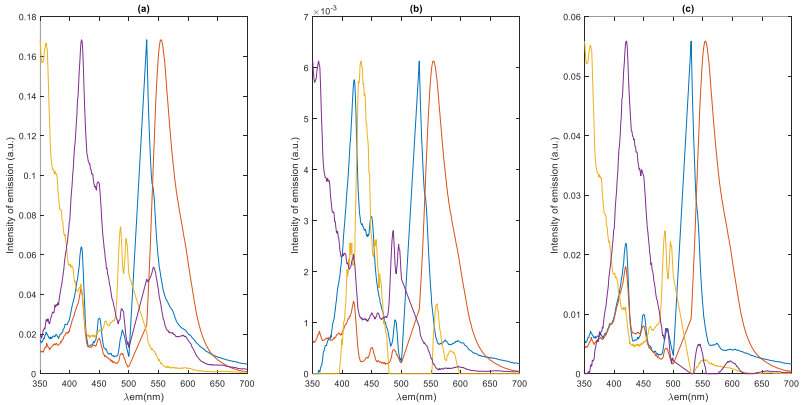


Figure 17: Resolved fluorescence emission spectra after applying correlations. (a) Correlations with Pink; (b) Correlations with Cherry Red; (c) Correlations with Incense Purple

Resolved concentration profiles for each dye and the theoretical profile can be seen at Figure 18.

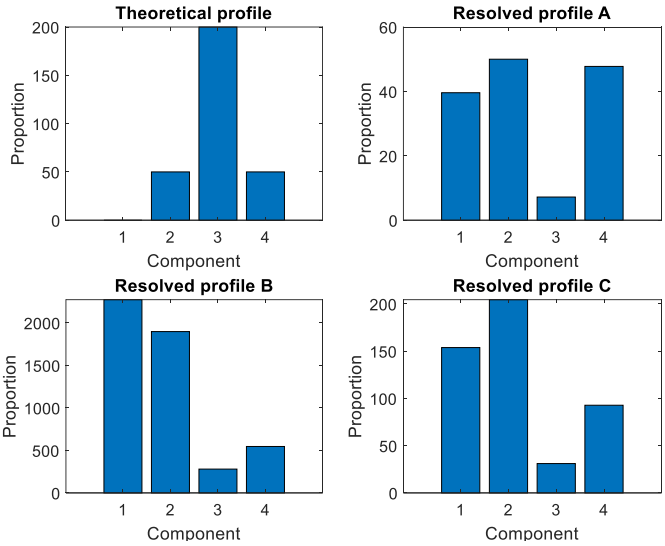


Figure 18: Comparison between a theoretical concentration profile and the profiles obtained after appliying correlations

In the case of the model where all the food colourings are analysed simultaneously, Figure 19, it is observed that there is more variability in the correlation and, the error is greater, since the values of RMSEC are higher than in the latter cases (Appendix 3). Thus, to predict the concentration of the food dyes on different mixtures with MCR-ALS using correlations, it would be better to build an individual model for the dye to quantify.

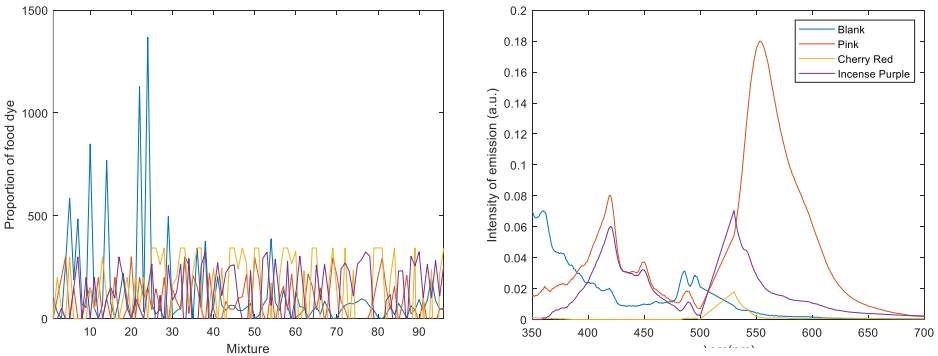


Figure 19: Optimised matrices after applying correlations. (Left) Resolved concentration profiles; (Right) Resolved fluorescence emission spectra

8.2. DATA ANALYSIS FOR 3D SPECTRA

On the other hand, 3D measurements were done, and the matrices obtained have been analysed. In this case, previous corrections were necessary since scattering peaks were visible, as seen in Figure 20.

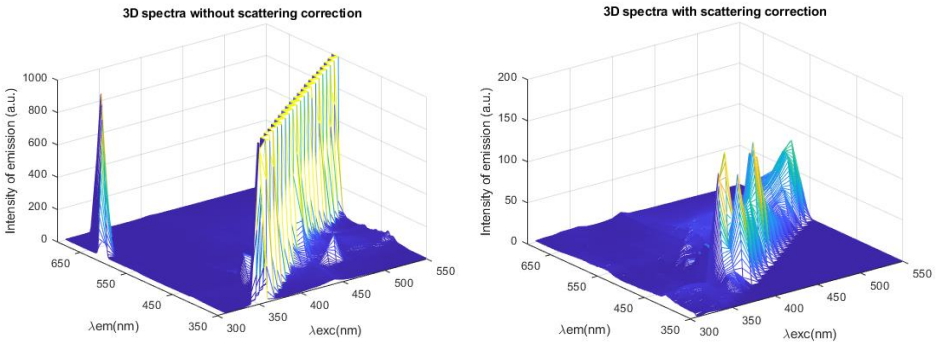


Figure 20: (Left) Example of 3D spectra without correction; (Right) Example of 3D spectra with correction

For the data corrected, other strategies were applied. On one hand, the data obtained was analysed with MCR-ALS only applying the constraint of non-negativity. On the other hand, another approach used is the Identification of Species, where on the first 24 rows of the matrix, species on each mixture were identified by presence (1) or absence (0) and the rest was identified with a presence of food dye, to identify if the model was capable of assign correctly the concentration profiles. In this case, since on each well a matrix is obtained, the initial estimation was the emission spectra at the λ_{exc} of each food colouring. The matrix to analyse is a sum of 96 individual matrices. Each individual matrix corresponds to a 3D spectrum of each well. Therefore, it must be specified this condition while applying the constraints. However, same constraints will be applied to all submatrices.

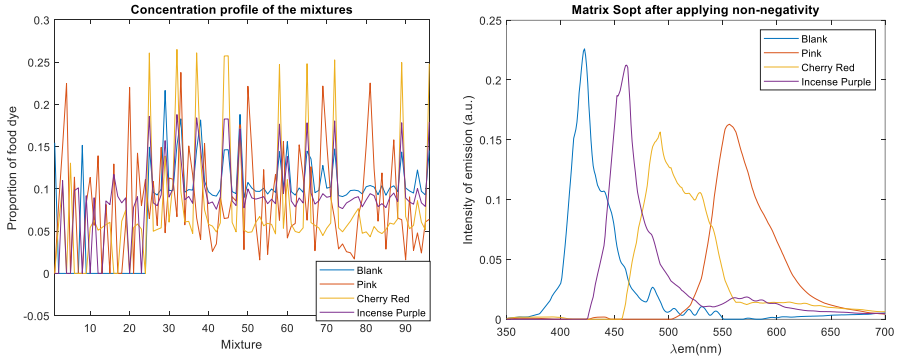


Figure 21: Optimised matrices after applying non-negativity. (Left) Concentration profiles; (Right) Resolved fluorescence emission spectra

It is observed in Figure 21 that the resolution of the mixtures does not improve. In this case, the blank presents a great signal, and its concentration profile can be greater than the other food colourings, even the food colouring Pink which on the latter cases it was well resolved. Moreover, the peak of emission of Cherry Red does not match entirely to the previous data observed, especially the spectrum obtained with a quartz cuvette.

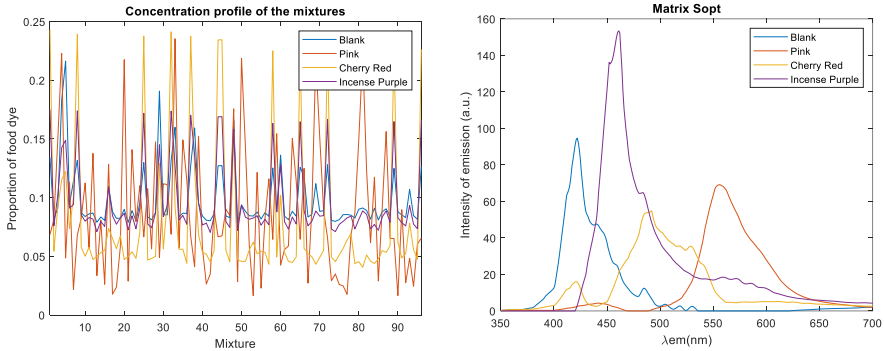


Figure 22: Optimised matrices after applying Identification of Species. (Left) Concentration profiles; (Right) Resolved fluorescence emission spectra

In the case of resolving the mixtures applying the Identification of Species, Figure 22, it is observed that the concentration profiles don't match the theoretical ones. This is mainly observed on the mixtures where the absence or presence of food dye were not specified, which are mixtures from 25 to 96. This means that the absence or presence of each food dye in a mixture should be specified on all samples, which is not favourable in case there are mixtures where the proportions of each dye are not known.

9. CONCLUSIONS

Once the project is completed, it can be concluded that the main objective, which is to evaluate the measurements of mixtures of food dyes through fluorescence spectroscopy and analyse the data through chemometric methods, was successfully accomplished. The minor goals were also completed successfully, and some conclusions can be drawn on the different studies.

Most of the studied samples presented fluorescence signal. All the samples were grouped according to the region where presented fluorescence excitation, that is at 300 nm or 500 nm.

Differences between measuring fluorescence emission with a quartz cuvette and a well-plate were observed too. The main difference between these two sample conditions was that the first did not suppose an interference on the measurements whereas the second interferes on the signal of fluorescence emission where most food dyes presented signal. Thus, optimizing the conditions was necessary because further measurements were carried out using well-plates as it produces faster results. The optimum conditions of measuring fluorescence emission in a well-plate were a volume of sample of 300 µL and a concentration of sample of 150 µL of sample diluted with 3000 µL of water. The plate that presented favourable spectra was a white well-plate.

Then, mixtures of food dyes were studied by means of the MCR-ALS chemometric methods considering different constraints. The data analysis led to the following conclusions:

- Firstly, four components were necessary on all the analysis made. This numbers corresponded to a component for each dye that makes up the mixture plus an additional component that corresponds to the well-plate (showing an emission signal which should not be ignored).
- In contrast, out of all food dyes analysed, the food dye that was better resolved overall, both on concentrations and spectra, was the food dye Pink.
- Out of all tested MCR-ALS constraints, the constraint that explained the mixtures better was the correlation constraint when applied to the 2D spectra data, especially when models were built for each single food dye.

- 3D excitation emission fluorescence data was also analysed. The results were not as favourable as the results obtained for the same mixtures with the 2D spectral data with correlation constraint. That is because the resolved concentration profiles did not match entirely to the theoretical concentration profiles of each food dye and this tendency was observed in all the models built. Building an additional model using correlations for 3D excitation emission fluorescence data could be an appropriate way to describe the data, like with 2D spectral data.

Finally, to consider the studied method as a possible way to quantify food dyes in the food industry the results obtained should describe the reality successfully. In this project was observed that despite the advantages of fluorescence spectroscopy, if the process happens to be implemented for routine analysis, the use of a well-plate is necessary. However, it was observed that the well-plates used were an interference when doing fluorescence measurements on most dyes, which was confirmed with further MCR-ALS analysis. But there are also food dyes that can be measured through the method studied successfully. Thus, it can be applied to these dyes, which can be a drawback because it is only applicable to a limited number of food dyes. However, if this method is further applied for the quantification of dyes, some improvements on experimental conditions should be done. An example could be using a well-plate that does not interfere with the fluorescence signal of the food dyes.

10. REFERENCES AND NOTES

1. Oplatowska-Stachowiak M, Elliott CT. Food colors: Existing and emerging food safety concerns. Crit Rev Food Sci Nutr. 2017 Feb 11;57(3):524–48.
2. WHO. Food additives. https://www.who.int/news-room/fact-sheets/detail/food-additives. 2018.
3. Carocho M, Barreiro MF, Morales P, Ferreira ICFR. Adding molecules to food, pros and cons: A review on synthetic and natural food additives. Vol. 13, Comprehensive Reviews in Food Science and Food Safety. Blackwell Publishing Inc.; 2014. p. 377–99.
4. Artificial food colours and children Why we want to limit and label foods containing the "Southampton Six" food colours on the UK market post-Brexit [Internet]. 2020. Available from: www.firststepsnutrition.org
5. Amchova P, Kotolova H, Ruda-Kucerova J. Health safety issues of synthetic food colorants. Regulatory Toxicology and Pharmacology. 2015 Dec 1;73(3):914–22.
6. Fallah S, Lützow M, Reinhart A. Safety of Colouring Foods-Regulations, Facts and Perceptions [Internet]. Available from: https://natcol.org
7. Tsai CF, Kuo CH, Shih DYC. Determination of 20 synthetic dyes in chili powders and syrup-preserved fruits by liquid chromatography/tandem mass spectrometry. J Food Drug Anal. 2015 Sep 1;23(3):453–62.
8. Januschewski E, Bischof G, Thanh BN, Bergmann P, Jerz G, Winterhalter P, et al. Rapid UV/Vis Spectroscopic Dye Authentication Assay for the Determination and Classification of Reactive Dyes, Monascus Pigments, and Natural Dyes in Coloring Foodstuff. J Agric Food Chem. 2020 Oct 21;68(42):11839–45.
9. Llamas NE, Garrido M, Nezio MS Di, Band BSF. Second order advantage in the determination of amaranth, sunset yellow FCF and tartrazine by UV-vis and multivariate curve resolution-alternating least squares. Anal Chim Acta. 2009 Nov 23;655(1–2):38–42.
10. Chfanou O, Tshldou M. Evaluation of the colouriug strength of saffron spice by UV-Vis spectrometry. Vol. 51, Food chemistry. 1996.
11. Ryvolová M, Táborský P, Vrábel P, Krásenský P, Preisler J. Sensitive determination of erythrosine and other red food colorants using capillary electrophoresis with laser-induced fluorescence detection. J Chromatogr A. 2007 Feb 9;1141(2):206–11.
12. Foruzin LJ, Rezvani Z, Nejati K. Preparation of two-color photoluminescence emission based on azo dye-layered double hydroxide systems and controlling photoluminescence properties of Allura Red AC. Journal of the Iranian Chemical Society. 2018 Dec 1;15(12):2649–58.
13. Yoshioka N, Ichihashi K. Determination of 40 synthetic food colors in drinks and candies by high-performance liquid chromatography using a short column with photodiode array detection. Talanta. 2008 Feb 15;74(5):1408–13.

14. Petigara Harp B, Miranda-Bermudez E, Barrows JN. Determination of seven certified color additives in food products using liquid chromatography. J Agric Food Chem. 2013 Apr 17;61(15):3726–36.
15. Dong MY, Wu HL, Long WJ, Wang T, Yu RQ. Simultaneous and rapid screening and determination of twelve azo dyes illegally added into food products by using chemometrics-assisted HPLC-DAD strategy. Microchemical Journal. 2021 Dec 1;171.
16. Wu W, Liu S, Guo T, Han X, Xia B, Wan Y, et al. Rapid screening of 70 colorants in dyeable foods by using ultra-high-performance liquid chromatography–hybrid quadrupole–Orbitrap mass spectrometry with customized accurate-mass database and mass spectral library. Food Chem. 2021 Sep 15;356.
17. Tang B, Xi C, Zou Y, Wang G, Li X, Zhang L, et al. Simultaneous determination of 16 synthetic colorants in hotpot condiment by high performance liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci. 2014 Jun 1;960:87–91.
18. Lakowicz JR. Introduction to Fluorescence. In: Principles of Fluorescence Spectroscopy. 3rd Edition. New York: Springer, cop. 2006; 2006. p. 1–26.
19. BMG Labtech. Fluorescence intensity [Internet]. Fluorescence intensity. [cited 2023 Jun 11]. Available from: https://www.bmglabtech.com/en/fluorescence-intensity/
20. Tauler R, de Juan A. Multivariate Curve Resolution for Quantitative Analysis. In: Data Handling in Science and Technology. Elsevier Ltd; 2015. p. 247–92.
21. De Juan A, Jaumot J, Tauler R. Multivariate Curve Resolution (MCR). Solving the mixture analysis problem. Analytical Methods. 2014 Jul 21;6(14):4964–76.

11. ACRONYMS

WHO: World Health Organization

UV-Vis: Ultraviolet-Visible

HPLC-DAD: High Performance Liquid Chromatography with Diode Array Detection

HPLC-MS: High Performance Liquid Chromatography joined to Mass Spectroscopy

Exc: Excitation

Em: Emission

MCR-ALS: Multivariate Curve Resolution-Alternating Least Squares

a.u.: Arbitrary units

C: Matrix of resolved concentrations

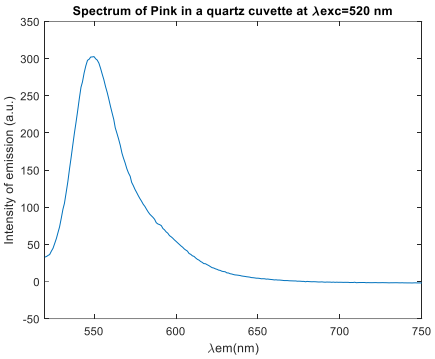
S: Matrix of resolved spectra

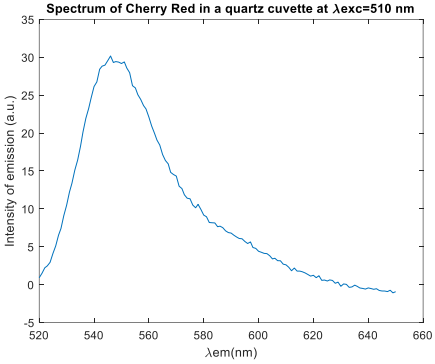
Copt: Matrix C optimised

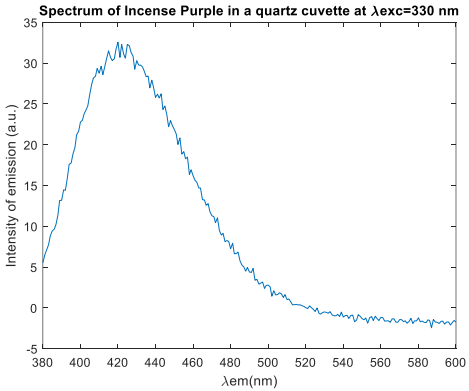
Sopt: Matrix S optimised

APPENDICES

APPENDIX 1: SPECTRA OF PINK, CHERRY RED AND INCENSE PURPLE IN A QUARTZ CUVETTE



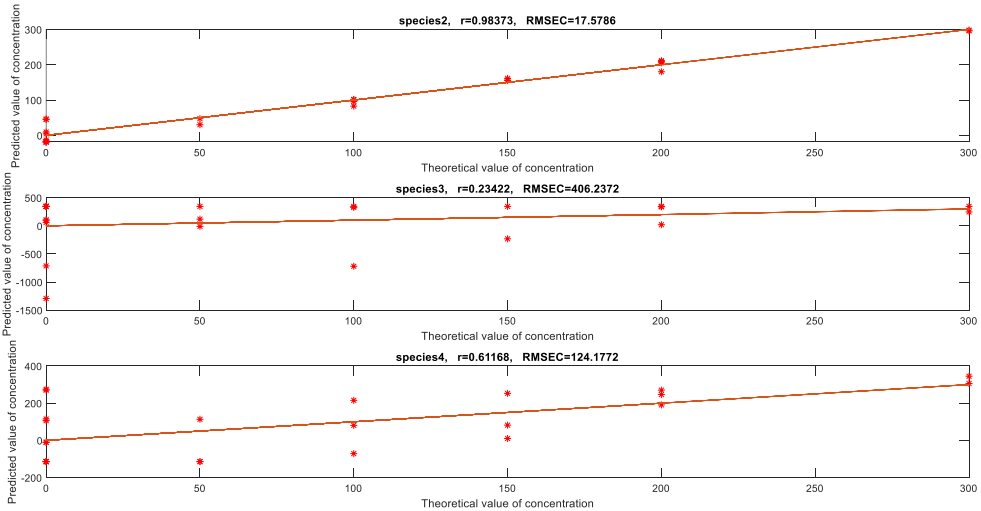




APPENDIX 2: PROPORTIONS OF DYES ON THE MIXTURES ANALYSED

Wells	H_20 (µL)	Pink (µL)	CR (µL)	IP (µL)
A1, H1, A4, H4, E5, D6, E6, B8, A9, H9, A12, H12	300	0	0	0
D1, D3, A5, B7, E9, A11	0	300	0	0
E1, E2, B6, D8, B10, E12	0	0	300	0
G1, A3, F5, E7, E10, B12	0	0	0	300
B2, B4, D5, A8, H8, D11	0	150	150	0
F1, H3, F4, F7, B9, B11	0	150	0	150
G2, E4, A6, C9, C10, D12	0	0	150	150
D2, D4, C7, H10	0	200	100	0
C1, F3, 5C, H6, C8, F9	0	200	50	50
H2, G5, G8, G11	0	200	0	100
F2, G4, F6, H7, D9, C12	0	100	100	100
B1, G3, G6, G10	0	100	200	0
A2, C6, G9, F11	0	100	0	200
C2, B5, D7, E8, F10, H11	0	50	50	200
C3, C4, H5, F8, C11, G12	0	50	200	50
E3, A7, A10, E11	0	0	200	100
B3, G7, D10, F12	0	0	100	200

APPENDIX 3: CALIBRATION CURVES OF THE OVERALL MODEL USING CORRELATIONS



(Top) Food colouring Pink; (Middle) Food colouring Cherry Red; (Bottom) Food colouring Incense Purple.