FOODINTEGRITY

Ensuring the Integrity of the European food chain

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1. Introduction

The early detection of frauds in foods and food ingredients has become an even more important topic in the last decade. Many consumers lost faith in the food they were purchasing and the food industry recognised that more robust measures in terms of auditing and testing had to be put absolutely and quickly in place.

Testing methods for the food industry must be easy to use, rapid and low cost, ideally. Rapid and portable technologies, which enable authentication to make point of use decisions on the industry, are highly desired.

The latest non-targeted analysis approaches can be useful to investigate on situations where there is no clear evidence of specific markers related to a defined fraud.

In the present document there are reported a number of different complete case studies, which elucidate the potentialities of many, powerful emerged and emerging analytical techniques towards the rapid (or rapid combined with confirmatory strategies) solution of frauds and adulterations issues associated to a wide range of food chains.

Samples involved in the studies were obtained directly from industrial sources as well as retail and service sectors and, when possible, assessment of the developed methods/technologies were carried on in the reception line and/or at the facilities of industrial production sites.

Data fusion & multivariate data analysis were also applied in order to increase the effectiveness of some approaches, especially with the aim to increase the confirmatory outcomes; at the same time the potential for further development into industrial on-site tests using handled instrumentation was taken into account.

2. List of Case Studies

- **RPA technology for tuna authentication on the canning production line**
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CASE STUDY 1 FOODINTEGRITY: WP10 AZTI (RAPID)

RPA technology for tuna authentication on the canning production line

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Background

During last years, canning industry has led to a processing revolution, since canned products are produced, in many cases, using imported frozen tuna fillets or loins. These skinned fillets offer tremendous advantages in terms of productivity and yield of the processes, yet on occasions, in view of the difficulty in distinguishing between species visually, errors may occur in the labelling of canned products. Therefore, the development of rapid assays for point of use authentication would allow the enforcement of labelling regulation; benefits the canning industry and finally the confidence of the final consumer. Tuna authentication methodologies are generally based on DNA fragment detection. These methods, known as genetic methods, are always very reliable but the downside is that it takes several days to obtain a conclusive result and it must be carried out by high qualified personal in control laboratories. These limitations have been overcome thanks to the innovative RPA (Recombinant Polymerase Amplification) technology[1] enables the identification of fish species in less than an hour, including DNA isolation step. This rapid and portable technology enables fish authentication to make point of use decisions on the industry.

Case Study: Yellowfin authentication assay on the canning production line

The yellowfin tuna (*Thunnus albacares*) is a worldwide distributed species which share the habitat with other tuna species such as the bigeye tuna (*Thunnus obesus*). As a matter of fact, both species are generally caught simultaneously and then processed together by the canning industry. In addition, real difficulties in identifying juvenile yellowfin and bigeye tunas do exist because these two species look very similar in their juvenile's stage that does happen, unfortunately, quite often.
The aim of this study is to assess the use of portable fluorimeter in the reception line at the facilities of a local canning industry using RPA technology. The feasibility of this study will be performed on imported frozen yellowfin loins.

Novelty statement

Until date, all the raw material control systems dealing with the detection of tuna species are based on DNA protocols that take several days. This study should demonstrate the use of RPA technology for the authentication of tuna species on the field.

Experimental

RPA assays were carried out in a total volume of 25 µL using the TwistAmp Exo kit (TwistDX, Cambridge, UK). Reactions contained 10 µM of specific reverse and forward primer, 10 µM of a specific TwistAmp Exo Probe, 0.1-10 ng of genomic DNA (isolated following previous protocol[2]), 14 mM of Mg acetate, and 1× rehydration buffer. First, all the reagents except for the DNA template and Mg acetate were prepared in a master mix, which was distributed into each 0.2 mL reaction tube containing the enzyme and the nucleotides in a dried pellet. Then, DNA was added into the tubes, and Mg acetate was dispensed lastly. Since the RPA reaction starts as soon as magnesium is added, the tubes were immediately placed into a portable T8 isothermal device (TwistDX, Cambridge, UK) at 39ºC for 15-20 min. TwistAmp Exo probe presents homology to the target amplicon that contains an a basic nucleotide analogue (a tetrahydrofuran residue) which replaces a nucleotide in the a target sequence flanqued by a

dT-fluorophore (FAM) and a corresponding dT-quencher group (these replacing T sequences found with the corresponding target sequence). In addition, probe are blocked from any potential polymerase extension by a suitable 3'-modification group (Spacer-C3). The design of specific probes and primers were done following the considerations of manufacturer.

Fig. 1. Portable T8 isothermal device (TwistDX, Cambridge, UK)

Results & Discussion

This study investigates RPA technology to discriminate between two closely-related Thunnus species target DNA molecules with a pair of primers, that amplifies a short diagnostic DNA fragment belong to mitochondrial cytocrome b gene DNA fragment, and a specific fluorescently labelled TwistAmp exo probe. The specificity and reproducibility of the system has been successfully evaluated. Fig 2 shows the specificity of the innovative RPA probe to discriminate between *T. albacares* and *T. obesus*.

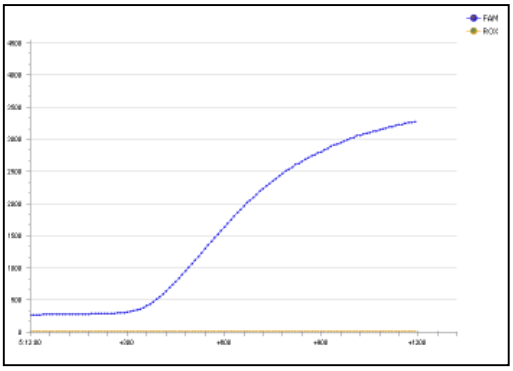

Fig. 2. RPA amplification curve. FAM fluorescence indicates the target amplification of T. albacares (blue curve). T. obesus were not amplified (yellow curve)

Final Outcomes

The results suggest that RPA technology is suitable to discriminate both tuna species within 15 minutes (excluding DNA isolation) with an inexpensive, portable and very simple to use small equipment, with little or no hardware required. This technology could be used in-, on-, by- or at-line in various locations of the food supply chain for control the integrity of food products.

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[1]Olaf Piepenburg, Colin H. Williams, Derek L. Stemple, and Niall A. Armes, 'DNA Detection Using Recombination Proteins'. *PLoS Biology,* 4 (2006), e204.

[2]Pardo, M.A. and B. Pérez-Villareal, Identification of commercial canned tuna species by restriction site analysis of mitochondrial DNA products obtained by nested primer PCR. *Food Chemistry*, 2004. 86(1): p. 143-150

CASE STUDY 2 FOODINTEGRITY: WP10 AZTI (RAPID)

Visible-Near InfraRed Spectroscopy (Vis-NIRS) application for differentiation of fresh and frozen/thawed tuna

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Background

The fillet of fresh tuna is an expensive product sold on local and international market. When fished at locations distant to market whole fresh tuna is frozen at temperature below -60 ºC to extent its shelf-life and it is sold as a frozen or frozen/thawed product. However, sometimes a fraudulent practice is found in the market when fillets or loins from thawed tuna are sold as fresh at a higher price. When freezing and thawing operations are carried out at proper conditions it is difficult to differentiate between fresh and frozen/thawed fillets.

Visible-near infrared spectroscopy (Vis-NIRS) is a spectroscopic method that uses the visble and near-infrared region of the electromagnetic spectrum (from about 415 nm to 925 nm). Typical applications include pharmaceutical, medical diagnostics, food and agrochemical quality control, and combustion research. During the last years, the use of Vis-NIRS is increasingly widely used in agriculture products and food quality evaluation because it meets the criteria of being accurate, reliable, rapid, non-destructive, and inexpensive[1].

In this work, we investigate the ability of Visible-Near InfraRed Spectroscopy (Vis-NIRS) to detect whether a sample of tuna is fresh or whether it has been frozen/thawed.

Case Study: Differentiation of fresh and frozen/thawed red tuna fillets

The ability of Visible-Near InfraRed Spectroscopy (Vis-NIRS) to detect the effect of freezing/thawing applied to fillets of red tuna (*Thunnus thuynnus*), contributing to the development of a non-invasive method for detection of the freshness of tuna fillets will be addressed. At the same time, the potentiality of this methodology for on-line identification of thawed tuna fillets will be also evaluated.

Novelty statement

This study should demonstrate the use of Vis-NIRS for the identification of the difference between fresh and frozen/thawed fish samples.

Experimental

As first step, in this investigation we compared Vis-NIR spectra collected from tuna samples before and after being frozen/thawed. The samples were obtained by cutting off 15 fillets (790g ±215g) from different tuna fish, in pieces (67g±30g). Thus, the obtained samples or pieces varied in size and fat content. By using this procedure we expected to produce a variation on the effect of freezing across samples, which would allow investigating Vis-NIRS in a more challenging situation. However, this could also mean that the effect of freezing could be affected by sample to sample variation (e.g. sample size). To deal with this type of problem it has been proposed the use of Multi-Level Partial Least Square-Discriminant Analysis (MLPLS-DA), which allows separating the effect of treatment from variation among samples. Thus we applied MLPLS-DA and Partial Least Square-Discriminant Analysis (PLS-DA) to evaluate ability of Vis-NIRS to detect whether a sample of tuna is fresh or frozen/thawed.

Vis-NIR data were collected in two sub-sets. In the first one (sub-set 1, n=12 fillets) each fillet was taken from 4 °C to a room at 16 °C, and cut into 9 pieces, which were covered with plastic film (the fresh cut

surface called 'T' was left upwards), and left for one hour in the room until they were scanned at 12.6±1.8 ºC. For three additional fillets (sub-set 2, n=3 fillets), similar procedure was used with a slight modification: after the fillet was cut into 9 pieces, they were left at room temperature (16 °C) covered with plastic film for one hour and then, a 5 mm slice of the transversal area was cut off for each of the nine pieces, which were left for another hour covered with plastic film at 16 ºC, and they were scanned at 17.4±0.5ºC. The modification on the procedure for sub-set 2 was applied to generate a set of samples with a higher temperature for the fresh samples on Vis-NIRS scanning. Vis-NIR spectra were collected on the original external surface (called 'S') and on the transversal surface ('T'), generated by cutting, for each of the nine pieces. After collecting the spectra, the samples were wrapped and frozen at -80 °C. After four days three samples of each fillet were transferred to 4 °C and left to thaw for twenty four hours. Then, the samples were transferred to a room at 16 °C and left to equilibrate for one hour, where they were unwrapped and weighted, and a slice of five millimetres was cut off from the same traversal section which was scanned by Vis-NIRS when fresh. The samples were then left for 1 hour with this fresh surface upwards and covered with plastic film. Afterwards, Vis-NIR spectra were collected on surfaces 'S' (original external surface of the samples) and 'T' (fresh/bloomed surface) at 17.5±1.4 and 17.8±1.0 ºC for subset 1 and subset 2 respectively.
The NIR equipment used to collect the data is composed by a spectrometer (AvaSpec 2048, Avantes, Netherlands), a light source (AvaLight-HAL,Avantes, Netherlands) and a fibre optic probe (FCR-7UVIR400-2-2.5x100,Avantes, Netherland), which is composed of 7 optical fibres of 200µm core (6 illumination-fibres and one read fibre). The reflectance spectra acquired is integrated in AvaSoft 8 software (Avantes, Netherlands).

Results & Discussion
Double cross validation (DCV) was applied to select the parameters of the classification models (*Filzmoser et al. 2009, Westerhuis et al. 2010, E. Szymaníska et al. 2012*). In double cross validation procedure the data set is first split in 'ndcv' sets (ndcv=3): one is used as test data set and the others are combined and used as calibration data set. The calibration data is used to fit a model. To fit this model it is necessary to identify the number of latent variables, which is done using cross validation procedure. In this case, the calibration data set is subdivided in 'ncv' sub-sets (ncv=4). The number of latent variables is increased from 1 to a maximum number 'nlvmax' (nlvmax=15). For each number of latent variables (1 to nlvmax) ncv models are fitted, by leaving each of ncv sub-sets out of the model fitting, and using the model fitted without that set to predict the data from the sub-set left out. Each sub-set is left out once, and at the end the predictions of the sub-sets which were left out are combined and the performance of the model is evaluated using the number of misclassified samples. The number of latent variables corresponding to the best performance (the minimum number of misclassified samples) is chosen. Then, a model is fitted with all samples from the calibration data set using the chosen number of latent variables and it is applied to the data from the samples of the test data set. This process is performed until all the ndcv sets have been used as test data set once. Double cross validation is repeated twenty times and the average of the twenty predictions for each sample is presented. In this study the procedure was repeated three times, for each time only one sample per fillet was used. This procedure was applied for MLPLS-DA and PLS-DA in similar subsets.
MLPLS-DA and PLS-DA were carried out using toolbox from Biosystems Data Analysis Group from the University of Amsterdam (*MLPLSDA*) using Matlab R2013a (Version 8.1.0.604, The MathWorks, Inc.). Data visualization was carried out in R v 3.2.0 (*R Core Team, 2015*) with package 'lattice'.
The results of prediction for MLPLS-DA and PLS-DA are presented in Figure 1. In this case, predictions for fresh ('Frsh') samples are expected to be lower than zero and samples that had been frozen/thawed ('Frzn/Thwd') higher than zero. Figure 1 shows that both models are able to detect the difference between fresh and frozen/thawed samples. However, PLS-DA seems to be affected by the 'initial' state of the sample, which is suggested by the presence of offsets in the predictions of fresh samples. This is corrected by MLPLS-DA which considers the same sample as control and treatment. There were two sub sets of the samples (sub-set 1 and 2), which present different scanning temperatures for the fresh samples (12.6±1.8°C/17.5±1.4°C and 17.4±0.5°C /17.8±1.0°C). There is no indication that the

temperature at time of scanning affected the predictions of MLPLS-DA as shown on Figure 1, where predictions for sub-set 1 (lower scanning temperature at fresh) are shown on the bottom row and for sub-set 2 (higher scanning temperature at fresh) are shown on the top row.

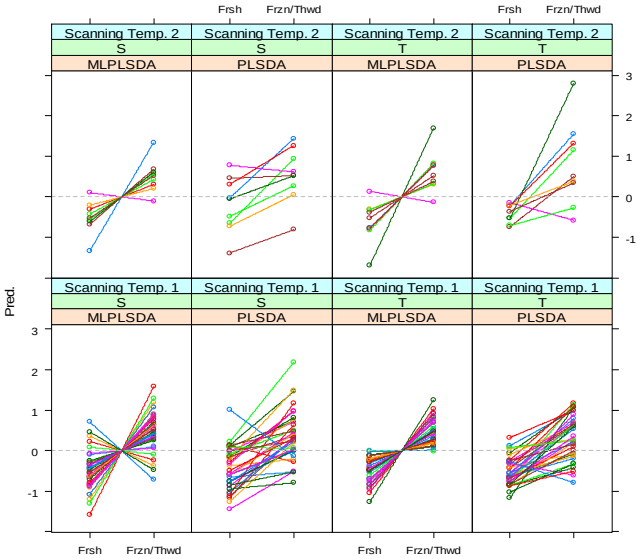

Fig. 1 – Predictions of MLPLS-DA and PLS-DA fitted to differentiate between fresh (Frsh) an frozen/thawed (Frzn/Thwd) tuna fillets. Predictions for fresh are expected to be lower than zero and for frozen/thawed higher than zero. Each line corresponds to one sample connecting prediction when fresh with prediction after being frozen/thawed. 'S' and 'T' represent the surface where Vis-NIR spectra were collected.Table 1 – Performance based on the number of misclassified samples for predictions of models MLPLS-DA and PLS-DA fitted to differentiate between fresh and frozen/thawed tuna fillets.

	Surface	NER	Sensitivity(fresh)/ Specificity(frozen/thawed)	Sensitivity(frozen/thawed)/ Specificity(fresh)
MLPLS-DA	S	87%	87%	87%
	T	96%	96%	96%
PLS-DA	S	78%	78%	78%
	T	81%	91%	71%

Sensitivity/Specificity: percentage of positives/negatives correctly identified. NER: Non-error rate.

Final Outcomes

Overall, the highest rate of correct classification is observed in spectra collected in surface 'T'. These results are an indication that Vis-NIRS is able to detect the difference between fresh and frozen/thawed samples.

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[1]**Burns, Donald; Ciurczak, Emil (eds.). Handbook of Near-Infrared Analysis, Third Edition (Practical Spectroscopy). pp.349–369.**

CASE STUDY 3 FOODINTEGRITY: WP10 UCPH (RAPID)

Elemental fingerprinting for authenticity testing of tomatoes using Laser Induced Breakdown Spectroscopy (LIBS)

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Background

Tomatoes are produced worldwide and are widely used in the diet as either fresh fruits or as processed products (sauces, purées and pastes). Several tomato products are sold at premium prices due to unique geographical origins or specific production methods (e.g. organic). Such products are at risk of food frauds and adulterations and several critical points in the tomato production chain call for introduction of efficient, high-throughput and low-cost analytical methods to reliably check the origin of raw materials.

Numerous studies have documented that soil mineralogy and fertilization strategy are key factors controlling the elemental composition of plant products. Consequently, elemental fingerprinting, often in combination with chemometrics, has been explored to enable verification of the geographical and agricultural origin of plant-based food products [1].

Elemental fingerprinting has also proven valuable for documentation of the authenticity of tomatoes and its derived products [2]. These studies have been based on multi-elemental analysis using various atomic spectroscopy methods. Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) does *e.g.* represent a sensitive analytical technique for analysis of most elements of the periodic table (even trace elements) but it relies on tedious and time-consuming sample preparations prior to analysis.

Laser Induced Breakdown Spectroscopy (LIBS) represents a rapid method for direct analysis of practically any material, as long as the laser it employs can generate a plasma on the surface of the material, and the spectrum of the light emitted from this can be recorded. The spectra obtained from LIBS contains information about the elemental composition of the sample and recent studies have demonstrated the applicability of LIBS for plant tissue analysis[3].

Case Study: Elemental fingerprinting of tomatoes using LIBS

The aim of the study was to explore the potential of LIBS as a high-throughput method for determining the geographical and/or the agricultural origin of tomato samples. We compared LIBS to another high-throughput method recently developed in our laboratory, *viz.* semi-quantitative (semi-Q) ICP-MS. Semi-Q ICP-MS requires acid digestion of samples and high-cost instrumentation for analysis. It was therefore hypothesized that LIBS may represent a cheaper and faster alternative for elemental fingerprinting.

Novelty statement

LIBS is potentially applicable for industrial implementation due to its minimal requirement for sample preparation and its ability to remotely analyse the multi-elemental composition of a sample from several meters of distance. It is expected that the developed methods will be applicable to several plant species and food matrices.

Experimental

The study was conducted on Italian tomato samples with a full provenance and traceability history. Samples (2 different varieties - Roma and San Marzano) were collected from Italian tomato growers (on-farm trials) in 2 geographical regions (Emilia-Romagna and Basilicata) in 2 years (2012-2013) from either organic or conventional growers (2 varieties x 2 regions x 2 years x 2 systems x 3 repetitions = 48 samples in total).

- *ICP-MS analysis*: samples of approximately 100 mg of plant material were digested in acid-washed microwave oven vessels containing 2.5 mL of 70% ultrapure HNO_3 (PlasmaPURE, 70%, SCP Science, Baie D'Urfé, Canada) and 1 mL of 15% H_2O_2 (30% Extra-Pure, Riedel de Häen, Selze, Germany).
  Digestion was performed in a pressurized single chamber microwave oven (UltraWAVE, Milestone Srl, Sorisole, Italy).
  The digested samples were diluted to 50 ml with ultrapure water (Milli-Q Element, Millipore) and stored at room temperature until analysis.
  Multi-element analysis was performed by semi-Q ICP-MS using a 8800 Agilent Triple Quadrupole ICP-MS (Agilent Technologies, Manchester, U.K.).
- *LIBS analysis*: dried and homogenised samples were pressed into cylindrical pellets using a 15 Ton Hydraulic KBr Press (PerkinElmer Inc.) and analysed by LIBS.
  The LIBS equipment used to perform all measurements consisted of an echelle-type spectrometer (Aryelle-Butterfly, LaserTechnik Berlin) with a 2048x512 pixel CCD-chip and a mechanical chopper to control the delay time between laser-firing and spectrum recording. This setup allowed for two spectral ranges to be observed, one from approximately 190 nm to 330 nm and the other from approximately 300 nm to 750 nm. The laser source was a Twins BSL 220 (Quantel) and an optical path (Fig. 1) consisting of three mirrors was used to guide the laser to hit perpendicularly onto the horizontal sample holder, and a 15 cm focal length lens was used to focus the laser onto the target.
  The sample holder consisted of an automated rotating disk upon which the target sample is placed and moved at a constant rate during measurements, thereby continuously exposing a fresh surface to the laser.

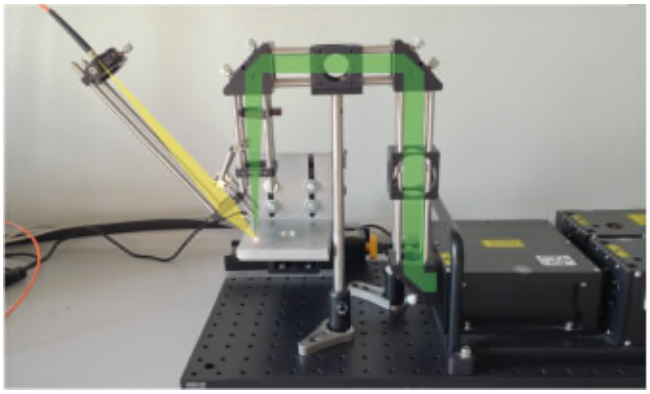

Fig. 1: LIBS setup used for tomato samples.

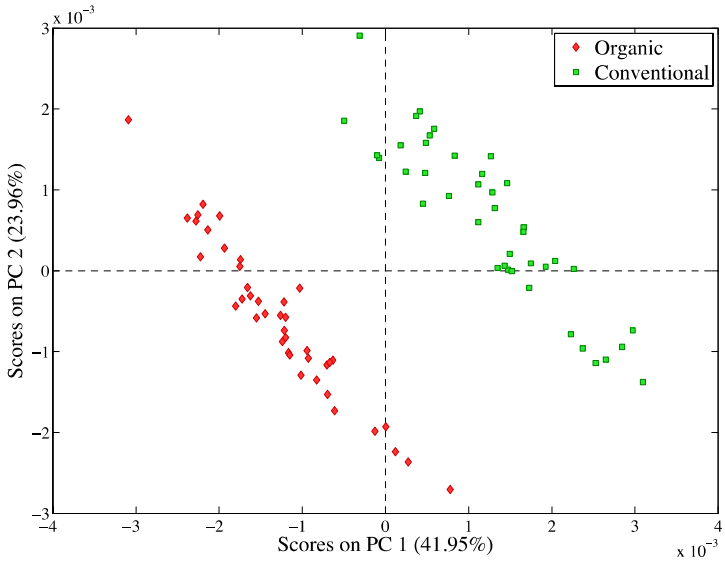

Fig. 2: Principal component analysis of LIBS data on tomato samples from 1 location (Emilia Romagna) and 1 growth year. .

Results & Discussion

The tomato samples from different Italian geographical origins, agricultural production methods and growth years could not be visually distinguished. The DM percentage did not differ either between tomatoes from the two geographical regions or the two investigated growth systems. Furthermore, the multi-elemental analysis by semi-Q ICP-MS did not reveal any single elements that varied systematically between growth locations and/or production system. However, when conducting multivariate statistics on ICP-MS data consisting of > 30 elements of the periodic table a clear effect of geography was observed across production systems and growth years.The initial multivariate statistics did thus not allow discrimination of organic and conventional tomatoes. However, when eliminating the effect of geography by focusing exclusively on tomatoes from Emilia Romagna, it was shown that organic and conventional tomatoes could be discriminated.

The observations were confirmed by the LIBS analyses. Multivariate statistics on LIBS spectra from all tomatoes did initially show a clear effect of geography and discrimination between organic and conventional tomatoes was not possible when including all tomato samples.

As observed for the semi-Q ICP-MS data it was necessary to focus on a single location in order to enable discrimination between organic and conventional tomatoes. When doing so, organic and conventional tomato samples from Emilia Romagna from one year were completely discriminated (Fig. 2).

Final Outcomes

It was shown that the geographical origin and the agricultural production method is reflected in the multi-elemental composition of tomatoes. This was documented by semi-Q ICP-MS on the basis of multivariate statistics of a data matrix consisting of > 30 elements of the periodic table. Elemental fingerprints were primarily affected by the geographical origin, however, when zooming in on a single location, discrimination between organically and conventionally grown tomatoes was feasible. Similar findings were observed for LIBS data. LIBS spectra were also dominated by the geographical origin but when focusing on a single location and one growth year the two agricultural production methods could be distinguished. It is thus concluded that elemental fingerprints obtained by either semi-Q ICP-MS or LIBS have the potential to determine where a plant has been grown when combined with chemometrics.

The agricultural production system was biased by geography but within a single geographical region organic and conventional tomatoes could be distinguished. In perspective, LIBS represents a major potential for industrial implementation However, a major challenge still lies in developing calibration procedures for LIBS and in the development of multivariate procedures for data analysis.

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[1] **Laursen, K.H., et al., Authentication of organically grown plants – advantages and limitations of atomic spectroscopy for multi-element and stable isotope analysis. *TrAC Trends in Analytical Chemistry*, 2014. 59: p. 73-82.**

[2] **Lo Feudo, G., et al., Investigating the Origin of Tomatoes and Triple Concentrated Tomato Pastes through Multielement Determination by Inductively Coupled Plasma Mass Spectrometry and Statistical Analysis. *Journal of Agricultural and Food Chemistry*, 2010. 58: p. 3801-3807.**

[3] **Santos, D., et al., Laser-induced breakdown spectroscopy for analysis of plant materials: A review. *Spectrochimica Acta Part B-Atomic Spectroscopy*, 2012. 71-72: p. 3-13.**

CASE STUDY 4 FOODINTEGRITY: WP10 CRAW-BARILLA (RAPID)

Case study for assessment of NIR Hyperspectral imaging to determine fraudulent adulteration of durum wheat

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Background
Durum wheat - DW (Triticum durum) is the main raw material in pasta production. Several major production countries, as Italy, France or Greece have decided that only pasta produced from DW is permitted and that the use of common wheat - CW (Triticum aestivum), should be considered as fraud. Since DW price (259€/T in May 2016) is higher than the CW price (157€/T in May 2016) in relation to the quality required by the pasta sector, the risk of economic fraud by mixing CW and DW is not negligible. According to current Italian rules, only a maximum of 3% CW is allowed to account for cross-contamination that may occur during the agricultural process. For the domestic market, and in countries where pasta may contain CW it has to be clearly indicated on the label the presence or not of CW. Efficient methods for the detection of accidental or intentional contamination of CW in DW products are therefore required.

Case Study: Discrimination between durum and CW by near infrared hyperspectral imaging
The current work, aims to develop a fast method for the at-line and on-line detection of CW grains in a lot of DW grains lot by NIR hyperspectral imaging spectroscopy. The spectral data treatment has been performed according to four approaches: morphological criteria (i), NIR spectral profile (ii), protein content (iii) and vitreous/not vitreous grains ratio (iv). Discriminant Chemometrics tools3 as Partial Least Squares Discriminant Analysis (PLS-DA) were applied in order to characterize DW and CW following each of the approaches. The results are presented at the grain level (4105 grains) and at the sample level (257 samples of +/- 4000 grains) based on the individual approaches or by combining the approaches.

Novelty statement
To date, all the studies dealing with the detection of CW in DW use macroscopic/ microscopic (morphological criteria)[1], chromatographic (HPLC on specific gliadin detection)[2] or molecular biology (real-time PCR for triticum species specific detection)[3] based methods. All these methods are confirmatory methods generally applied on semolina or bakery end-products. This feasibility study demonstrates the use of NIR as screening method for detecting CW grains in DW with potential use at the reception of the trucks in food companies.

Experimentals
To assess the possible mixture of cereals, 77 samples of DW and 180 samples of CW collected in Belgium and Italy in 2014, 2015 and 2016 were collected.
The samples of DW covered the quality variability at the collect of grains at the reception of Barilla Company and CRA-W allowed to collect a set of samples covering a large variability in terms of varieties.

Regarding the morphological criteria, the 8 following criteria have been studied:

Area: A= π*r2 ; area of grain (pixels2)
Perimeter: P=2π*r ; length of grain perimeter (pixel)
Circularity: C=4π*A/P2; perfect circle: C=1
MaxFeret = Feret's diameter of grain (length in pixels) = the longest distance between any two points along the particle boundary
MinFeret = Minimum Feret's diameter of particle (length in pixels)
AR (aspect ratio) = major_axis/minor_axis
Round (roundness) = 4*area/(π*major_axis^2)
Solidity = area/convex area

These parameters were calculated from the NIR-HIS images (Figure 2).

In order to study the composition and variability of the samples set, all of them were initially analyzed using a FOSS XDS NIR spectrometer active in the 400–2500-nm range. Quality parameters such as moisture, protein, and zeleny were estimated using equations constructed with historical NIRS databases[4]. The predicted values for the protein content were used to build PLS-DA models to discriminate samples at high protein content (HP: >12%) and low protein content (LP: <12%). Regarding vitreousness, 8 vitreous grains and 8 not vitreous grains were selected within each sample based on visual observation. Hyperspectral images are collected with a SWIR ImSpector N25E spectral camera from Specim Ltd using a cooled, temperature stabilized MCT (Mercury-Cadmium-Telluride) detector, combined with a conveyor belt (Burgermetrics).

The samples are spread on the conveyor belt manually or using a sample feeding system developed at CRA-W (Figure 1). One NIR image at grain level (16 grains) and +/- 30 NIR images at bulk level (200 g subsample) were acquired for each sample.

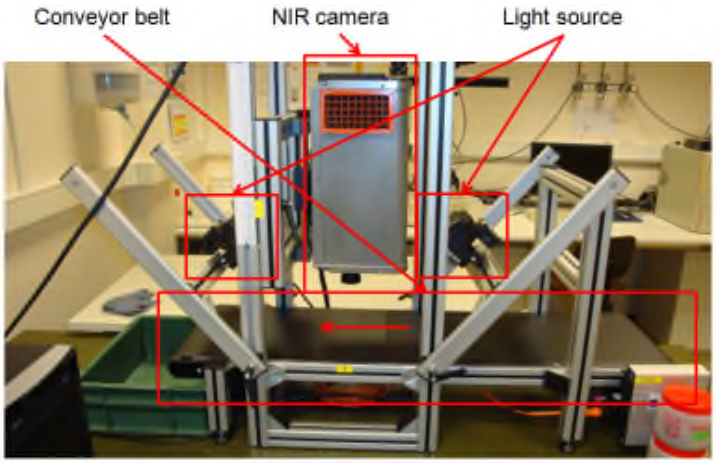

Fig. 1: NIR line scan imaging system using a sample feeding and a conveyor belt for analysis.

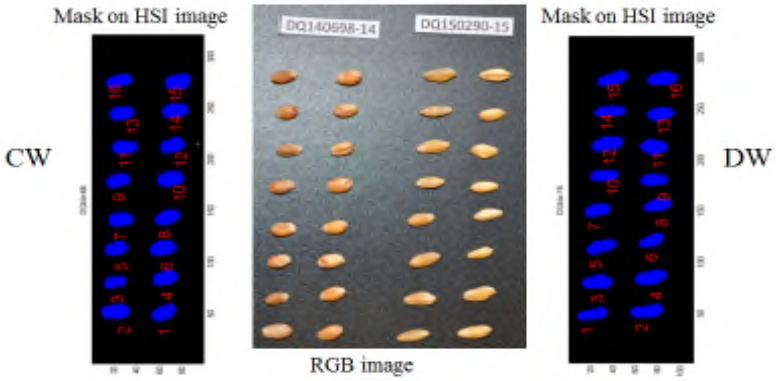

Fig. 2: RGB picture showing the grain presentation on the conveyor belt and mask on images after DBSCAN

The data treatment involved building libraries from images on 16 grains for each class of species, DW and CW. To extract the data from the image, a mask to isolate the grains was built by applying a Partial Least Squares Discriminant Analysis (PLS-DA) model combined with the density-based spatial clustering of applications with noise method (DBSCAN) procedure on each image.
To perform the discrimination models several strategies were defined to select the calibration and validation sets.
Then the models were applied on the fully independent samples sets: the CW2 set 2015 and all the sets 2016. For morphological criteria, the model was applied at grain level on the images of 16 grains. For spectral profile, the model was applied at pixel level on the images of 16 grains and a decision rule to determine the species was defined at grain level. For protein content and vitreousness, the models were applied at pixel level on the images of 200 g subsamples.

Results & Discussion

Models were developed on samples collected in 2015 (images of 16 grains) and validated on samples collected in 2016.
The final step of data elaboration was the data fusion techniques at grain level and sample level on all the results in order to improve the DW/CW discrimination performance by combining 2, 3 or 4 approaches together.
At the grain level, around 90% of grains are rightly classified based on the morphological criteria or the spectral profile. By combining these 2 criteria, 98.8% of DW grains are sorted in the cereal batch and 86.0% of CW grains are sorted out the cereal batch. The approach based on protein content gives less good results with right classification around 65%.
At the sample level, more than 95% of samples (mean of 16 grains) are rightly classified based on the morphological criteria or the spectral profile. By combining these 2 criteria, 100% of DW are sorted in the cereal batch and 100% of CW are sorted out the cereal batch. Regarding protein content and vitreousness assessed on samples of 200 g, 96% and 91% of DW samples respectively are sorted in the cereal batch. 82% and 88% of CW respectively are sorted out the cereal batch. By adding the protein content or the vitreousness criteria to morphological criteria or the spectral profile, 100% of right classification is also obtained for all samples.

Final Outcomes

This study shows the potential of NIR hyperspectral imaging combined with chemometrics to propose solutions for sorting grains at the entrance of the production chain according to the species (morphological and spectral profile), the protein content and the vitreousness.
These results show that the method based on the morphological criteria or the spectral profile allows to detect fraud with 100 % of right classification on subsamples as small as 16 grains. The models based on protein content and vitreousness less efficient to discriminate between DW and CW can be used to sort out low protein and low vitreousness batches inside the DW class by adjusting the thresholds.
The next step will be the validation of these approaches on other sources of CW and DW (France, Canada) as well as on real mixtures of DW and CW to challenge the detection of 3% of adulteration of DW with CW.

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CASE STUDY 5 FOODINTEGRITY: WP10 QUB (RAPID/CONF..)

Case study for assessment of FT-IR & Mass Spectrometry to determine fraudulent adulteration of Oregano

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Background

Oregano is a culinary herb most commonly associated with pizzas and other Mediterranean dishes. The main producers of oregano reside in the United States of America, Mexico, Greece and Turkey. Compared to most herbs, oregano has a complicated history as the true identity of it is very difficult to define. This is partly due to the large heterogeneity of the Origanum genus, but also due to the grouping of different botanical genera; Origanum (lamiaceae) from the Mediterranean and Lippia (verbenaceae) from Mexico.[1] Due to the confusion, this led to a clear market distinction between Mediterranean and Mexican oregano, with both having different cleanliness specifications such as the addition of sumac leaves.[2] Mexican oregano has a much stronger and robust flavour compared to Mediterranean oregano, which could be due to the varying percentages of essential oils within the leaves. The essential oil percentage in Mexican oregano leaves is around 3-4%, whilst the percentage within Mediterranean oregano leaves is around 2-2.5%. Even with the clear commercial distinction between Mexican and Mediterranean oregano, there are still several different definitions regarding Mediterranean oregano. The European Pharmacopoeia (PhEur) and the European Spice Association only allow Origanum vulgare L. ssp. hirtum and Origanum onites L., to be marketed as true oregano with impurities of extraneous materials of up to 2% being considered tolerable.[2]
However the herb and spice industry believe that oregano is being bulked by waste leaves such as olive and myrtle for which the industry use a microscopy test.

Case Study: development of FT-IR and LC-HRMS methods to determine fraudulent adulteration of oregano[3]

The aim of this study was to develop and fully validate a two-tier approach utilising Fourier-Transform Infrared spectroscopy (FTIR) and Liquid Chromatography High Resolution Mass spectrometry (LC-HRMS) to screen for and confirm oregano adulteration. When these two techniques are combined with multivariate data analysis software they have the ability to process a large number of samples. Samples will be obtained from industrial sources as well as the retail and service sectors. Potentially the tests could be further developed on handheld FT-IR for use in the field and ports of entry.

Novelty statement

To date there has not been a comprehensive strategy to screen for and confirm the adulteration of oregano with potential for further development into industrial on-site tests using handheld instrumentation.

Experimental

Samples of oregano with full provenance and traceability and a number of previously identified adulterants (olive leaves, myrtle leaves, sumac leaves, cistus leaves, hazelnut leaves), were sourced from different parts of the world. The samples were milled to a homogeneous powder on a PM-100 Retch Planetary Ball Mill (Haan, Germany) by weighing approximately 5g into grinding jars and milling at 500 rpm for 5 minutes.

For LC-HRMS analysis, milled homogenate herb sample (0.05g) was extracted in 2 mL of methanol/water solution (1:1, v/v), mixed for 10 minutes, sonicated for 15 minutes at maximum frequency in a water bath at room temperature, centrifuged at 10,000 g for 10 minutes at 4°C and the supernatant collected (1 mL). The supernatant was dried and reconstituted in 1.5 mL of ultra-pure water. Subsequently, the extract was filtered through a 0.22 µm Costar Spin-X Centrifuge Tube Filter (10,000 g at 4°C for 10 minutes). Filtered extracts were immediately transferred into Waters maximum recovery vials for UPLC-QTof-MS analysis.

- *FTIR analysis:* the milled samples were placed in the ATR sample area of a Thermo Nicolet iS5 spectrometer (Thermo Fisher Scientific, Dublin, Ireland) equipped with ATR iD5 diamond crystal and ZnSe lens and DTGS KBr detector. Each spectrum was acquired in the 550-4000 cm-1 range. Spectral data for each sample was averaged before further data processing.
- *LC-HRMS analysis*: analyses were carried out on a Waters Acquity UPLC I-Class system (Milford, MA, USA) coupled to a Waters Xevo G2-S QTof mass spectrometer (Manchester, UK).
  The chromatographic separation was conducted on an Acquity HSS T3 column (100 mm x 2.1 mm, 1.8 µm). The column oven temperature was set at 45ºC, injection volume at 5 µL and flow rate at 0.4 mL min-1. Mobile phase consisted of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid. The gradient was set as follows: 1.50 min of 99% (A) followed by a linear increase from 1 to 99% (B) over 15 min, isocratic cleaning step at 99% (B) for 2 min, then returned to initial conditions 99% (A) over 0.25 min and column equilibration step at 99% (A) for 1.25 min. Each sample was injected three times in order to assure reproducibility.

Principal Components Analysis (PCA), an unsupervised technique, and Orthogonal Partial Least Squares Discriminate Analysis (OPLS-DA), a supervised technique, were used for building the qualitative models in this investigation as previously described (Haughey et al, 2015).

Results & Discussion

In the FT-IR spectra of pure oregano, olive leaves and myrtle leaves spectra there are observable differences in the fingerprint region from 900-1800 cm-1. To further observe the influence of adulterants on the spectrum of oregano, the latter was adulterated in 10% additions (0-100%) of olive leaves and the spectra recorded. Figure 1 shows the resulting spectra indicating the monotonic increase intensity exemplified by the peak shown in the inset.

Due to these differences identified in the spectral data it was possible to apply chemometric modelling for discriminant analysis and the results obtained indicated that this method could rapidly screen for adulteration of oregano is sufficiently robust and thus fit for purpose.

After LC-HRMS analysis, up to 4500 ions in each ionisation mode were reliably detected along the chromatographic gradient (Figure 2)

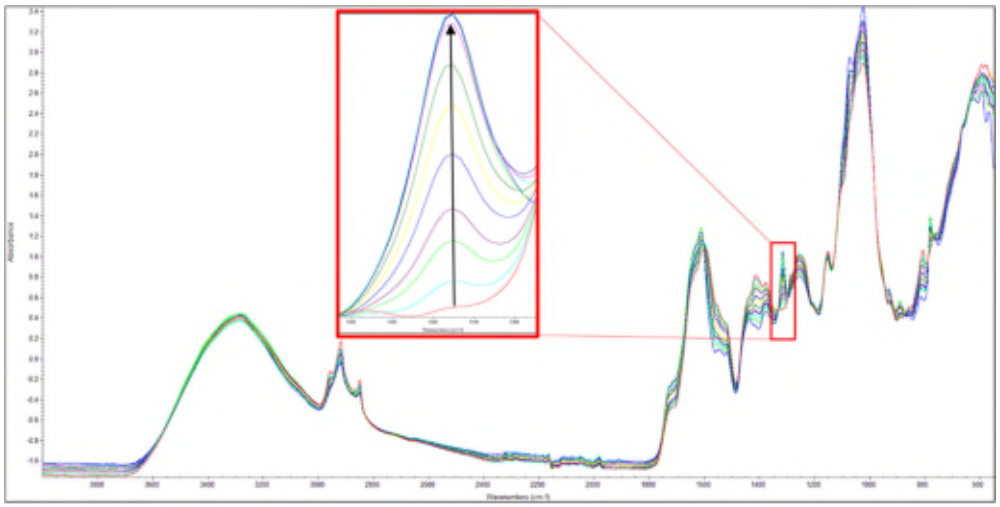

Fig. 1: FT-IR spectra of Oregano adulterated with olive leaves in 10% additions (0-100%) showing a monotonic increase in intensity exemplified by the inset with the arrow indicating the increase in olive leaf adulteration

The extracted data were then exported to chemometric software to be subjected to similar data treatment as was the spectroscopic data presented above.
The PCA score plot generated showed clear discrimination between the pure oregano and the adulterants, with the oregano samples clustered together on one side of the plot and the adulterant samples scattered on the other side of the plot.
After OPLS-DA elaboration, 16 unique markers in positive mode and 12 in negative mode were identified, with all adulterant samples having at least 4 unique markers. This data will be used for the future development of a targeted method using MS/MS analysis.

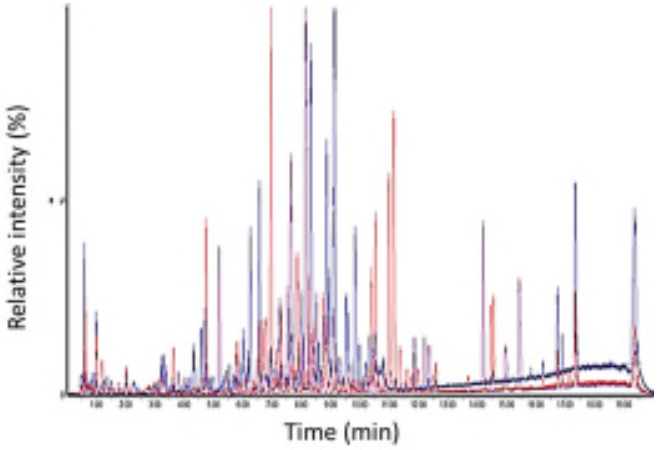

Fig. 2: overlay full scan chromatograms of oregano, olive leaves and myrtle leaves

To test the models developed for both analytical methodologies, a survey of oregano was carried out which included samples collected globally from retailers, service sector, internet sources with the help of Food Integrity partners. Samples continue to be sent for analysis from all around the world. The spectral data generated for these samples from the FTIR and LC-HRMS were predicted as unknowns using the relevant OPLS-DA model produced earlier.
Figure 3 shows the results of the survey which have been broken down by country where they were procured. The results show that approximately varying levels of oregano fraud depending on the source. The scale of adulteration ranged from 10% to over 70%, indeed several samples had virtually no oregano present. The scale and level of the adulteration uncovered was not expected. The most common adulterants found in the samples were olive leaves and myrtle leaves although samples from Australia, South Africa and USA were also found to contain cistus and/or sumac leaves.

Country	Samples Adulterated	
Canada	5/7	71%
South Africa	6/10	60%
Australia	7/12	58%
Portugal	2/6	33%
Germany	3/10	30%
Poland	3/10	30%
USA	7/28	25%
UK/Ireland	19/78	24%
Belgium	2/9	22%
France	2/12	17%
Norway	1/6	17%
Netherlands	2/18	11%
Italy	1/12	8%
Sweden	1/12	8%
Spain	0/5	0%

Fig. 3: global overview of oregano adulteration

Final outcomes

The detection of fraud in foods and food ingredients has become an even more important topic since the horsemeat scandal of 2013. Many consumers lost faith in the food they were purchasing and the food industry recognised that more robust measures in terms of auditing and testing had to be put in place. Often fraud is perpetrated in high value food commodities and those which come via complex supply chains. Probably herbs and spices fit these characteristics more than any other food ingredients and are thus highly vulnerable. Testing methods for the food industry must be easy to use, rapid and low costs. Our two tier system of testing provides not only a cost effective means of testing but one also that will survive rigours of a legal process. The survey data presented is disturbing in the level of adulteration found. It is clear that a serious level of fraud is being perpetrated and that bona fide businesses and consumers are being financially harmed. It is likely similar (if not worse) levels of fraud are occurring in many global regions. We believe the system we have developed and validated for oregano should be expanded to cover all herbs sold in the market. Only then will there be a sufficient deterrent in place to stop fraudulent activity in these widely consumed food ingredients.

One of the major outcomes of this study has been the global impact with headlines in many media outlets around the world. Figure 4 shows some of the headline news that was achieved through using our fraud test and the global survey.

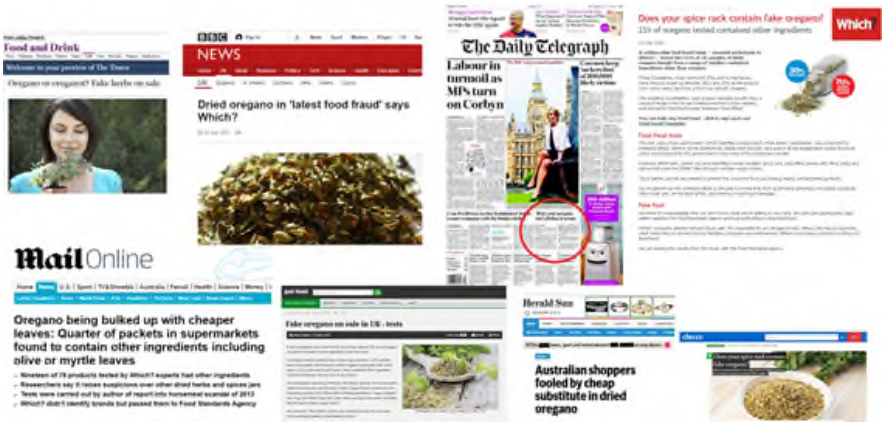

Fig. 4: Headline news indicating the global impact of the oregano fraud test and survey results

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CASE STUDY 6 FOODINTEGRITY: WP10 BARILLA (RAPID/CONF..)

Detection of emulsifiers used as unlabeled ingredients in finished products PASTA through both direct (e.g. LC-MS/MS) and indirect (e.g. XRF, enzymatic kits, free fatty acids GC-FID ratio) strategies

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Background

Pasta is a very simple product in ingredient composition, made only by semolina and water, but it is various for composition and legislation issues worldwide; most of the producers start from durum wheat varieties but also the market of soft wheat pasta is in expansion. From the organoleptic point of view, soft wheat pasta is not able to keep texture after cooking as well as durum wheat one; it is due to the real nature of the raw material: wheat with soft kernel is high in starch but usually low in gluten.
To reduce the cost of raw material and meanwhile to preserve pasta texture, sometimes durum wheat is mixed with the soft one without declaring it; in addiction, emulsifier agents (ex. stearoyl-lactilates) are employed without a declaration on the labels.
At the moment, the state of the art for identification and quantification of the fraud presented above is:

- For the detection of soft wheat it is already in use Real Time PCR technique; thanks to the high method sensibility it is also able to detect the natural "contamination" (about 5%) occurring from the supply chain[1].
- Other emulsifiers (i.e. Mono-Di glycerides), are detected and quantified with LC-MS/MS techniques[2].
- For both the emulsifiers of the stearoyl-lactilates class, at the moment it is not defined an analytical method able to discriminate whether the emulsifiers level is significant to attest their fraudulent addition or not.

The development of an authentication method of pasta real composition would allow the enforcement of labelling regulation, benefits the canning industry and therefore the confidence of the final consumer.

Case Study: detection of emulsifiers in finished product PASTA

Aim of this study is the development of methods able to detect the addition of unlabeled additives (E471 and E481/82) in finished PASTA.
The detection could be direct (this approach is not always possible due to target molecular weight) or indirect, detecting emulsifier fragments after pasta enzymatic treatment.

Novelty statement

This study should demonstrate that a simultaneously direct and indirect approach is able to highlight the fraudulent addition of emulsifiers (E471 and E481/482), coupling the results obtained with confirmatory techniques with the ones obtained with rapid screening techniques. These methods, if treated separately, are not able to give a complete overview of the problem.

Experimental

Different PASTA samples from the Brasilian market have been analyzed (all declared "pasta made with durum semolina"); a Barilla standard spaghetti sample (that doesn't carry emulsifier added) was used as negative sample. Moreover, a mixture of emulsifiers E481 have been added (1% of total amount) to this sample in order to have a certain positive sample.

For the detection of E471 (Mono-Di glycerides), an LC-MS/MS method was used. The E481/82 emulsifiers (Sodium and Calcium salts of stearate esters of lactic acid) were detected indirectly: a direct XRF measurement on the grinded pasta was performed in order to detect the presence of Calcium in the sample (XRF: Spectro IQ II Ametek, Kleve, Germany). Subsequently, after sample digestion with KOH, the ratio between stearic acid (C18:0) and oleic acid (C18:1) was evaluated using GC-FID (Trace 1310, Thermo Fisher Scientific, Waltham, Massachusetts, USA with Nukol capillary Column) and the Lactic Acid was quantified with an enzymatic kit (R-Biopharm, Darmstadt, Germany, test No.10139084035). The simultaneous detection of these three segments of the precursor molecule could strongly be correlated to the actual presence of the emulsifier in the sample.

Results & Discussion

To what concern the E471 emulsifier, all the samples gave results lower than the limit of detection of the applied method. For the E481/82, the XRF analysis highlighted that a group of samples (coming from the same supplier) had an high amount of Calcium. The same group of samples had also the highest values of free fatty acids (FFA) ratios and Lactic Acid amounts (the "SUPPL_A bavette" sample had even value comparable to the Barilla's spiked sample). The following table resumes the results obtained:

Sample Description	Ca mg/100g	C18:0/18:1	Lactic Acid mg/kg
SUPPL_A bavette	107	1.97	1500
SUPPL_A espaguete	49	0.54	840
SUPPL_A penne	37	0.54	830
SUPPL_A parafuso	37	0.29	640
SUPPL_B espaguete	19	0.25	726
SUPPL_C espaguete	19	0.08	674
Blank sample BARILLA		0.12	
Blank sample + 1% E481		2.08	

Tab. 1: results obtained for indirect analysis for E481/82 detection

According to these data, thanks to a cross comparison with Barilla spiked sample, we could declare that SUPPL_A added about 1% of Sodium Stearoyl-Lactylate to the bavette commodity.

Final Outcomes

For the first time an indirect method for the detection of E481/82 was presented. Moreover, this study highlighted that, thanks to the combined information collected by the smart application of different analytical techniques, the addiction of unlabeled ingredients can be detected; the union of several connected results showed clearly the adulteration of a sample and the same conclusion should not be reached with the same efficacy analyzing each method alone. This approach can be used during several steps of the production chain, increasing food quality and consumer satisfaction and safety.

[1] **Alary R. et al, Quantification of Common Wheat Adulteration of Durum Wheat Pasta Using Real-Time Quantitative Polymerase Chain Reaction (PCR). Cereal Chemistry, 2002. 79(4): p. 553-558**

[2] **Suman M.. et al, Determination of food emulsifiers in commercial additives and food products by liquid chromatography / atmospheric-pressure chemical ionisation mass spectrometry). Journ. of Chrom. A, 2009.1216: p. 3758-3766**