



FOODINTEGRITY

Ensuring the Integrity of the European food chain

613688: Collaborative Project

Seventh Framework Programme

KBBE.2013.2.4-01: Assuring quality and authenticity in the food chain

Deliverable D10.2

Title: *Testing and validation of profiling/fingerprinting/ targeted or non-targeted fundamental methods, selected/developed within the previous work-packages*

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Beneficiary(s): *Barilla SpA (Partners n°19 in the Food Integrity Consortium)*

Date of preparation: January 27th 2017

Period covered: October 2015 – December 2016

Status: Completed (Version 1)

Dissemination level		
PU	Public	
PP	Restricted to other participants	
RE	Restricted to a group specified by the consortium	
CO	Confidential, only members of the consortium	X



The project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement No. 613688.

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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.

On the other side, occasionally, food industry needs also to confirm the results through very reliable methods that has the downside to take usually long analytical /data interpretation time to obtain conclusive results and high-qualified personnel with expensive instrumentation in control laboratories.

Finally, 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 confirmatory 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 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.

2. List of Case Studies

- **Beef and pork species accurate quantitation in highly processed food matrix through LC-MS approaches.**
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Beef and pork species accurate quantitation in highly processed food matrix through LC-MS approaches.

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Background

Food authenticity currently represents an aspect of crucial importance inside the food quality. Usually it refers to the adherence to specific ingredients, to a specific origin of production or to specific technologies declared on the labels. Current issues in food authenticity, generally linked to economic reasons, are concerning mislabeling or food adulteration with lower cost materials. Adulterated food can be defined as food incompatible with the declaration of the seller. In the case of meat and meat articles, adulterations refer not only to the replacement of ingredients but also to inappropriate information concerning the origin of raw materials. Species authentication in processed meat is currently one of the most important authenticity related issues, in particular after the recent events regarding adulteration of meat products with non-declared species. At the present time, meat and meat product species investigation is mainly based on DNA detection or protein-based immunochemical methods (ELISA or LFD). Although these methods are very sensitive and specific, they have some limitations. In particular, they are mainly focused to identify traces of not expected meat species, but none of them is able to accurately quantify the percentage of specie in a mixture. Since the amino acid sequence of proteins is genetically determined, they essentially carry the same information coded in DNA, and the derived peptides, which are more resistant to technological treatment, can be useful markers to provide information on the ingredient used.

Case Study: Beef and pork accurate percentage quantitation in highly processed food matrix such as Bolognese sauce.

Bolognese sauce is one of the most difficult food matrixes to be analyzed, both for the heavy thermal treatment and for the high number of interfering ingredients. The quantification of pork and beef in the recipe will be achieved using different marker peptides released from meat proteins after driven digestion with selected enzymes. These peptides will be first characterized by LC-HRMS and then quantify using LC-LRMS method. Once set up, the method could be routinely applied to detect adulteration of meat-based preparations.

Novelty statement

Up to now, methods aiming at investigating meat species authenticity are based on the analysis of protein composition or on the analysis of nucleic acids, with several limitations. This study is aimed to propose an emerging tandem mass spectrometry method to detect beef and pork meat in very complex and heavily processed food matrices, such as ragout, both in qualitative than in quantitative way.

Experimental

One hundred grams of Bolognese sauce samples were homogenized and then lyophilized.

1 g of the lyophilized sample was added to a solution of 50 mmol L⁻¹ TrisHCl (pH 8), 6 mol L⁻¹ urea and 1 mol L⁻¹ thiourea and homogenized for 5 minutes. The resultant mixture was centrifuged and the supernatant was filtered through 0.45 µm syringe driven filters; protein concentration was measured according to the Bradford assay (Kruger, 2002).

Samples were desalted using Sep-pak Plus C18 cartridges (Waters, Milford, MA, USA), and the eluted fractions were dried.

Solid residues were reconstituted with NH₄HCO₃ 50 mmol L⁻¹; disulphide bridges were first reduced with dithiothreitol 200 mmol L⁻¹ and then alkylated with iodoacetamide 1 mol L⁻¹. After the addition of trypsin (enzyme to protein ratio 1:20) the digestion was carried out overnight at 37°C. Digested samples were then centrifuged and the resulting supernatant was then directly analysed by LC-MS.

The LC-LRMS study was performed with an UHPLC/ESI-MS system (Acquity Ultraperformance UPLC with a single quadrupole mass spectrometer, Waters SQD) on a RP column (Acquity UPLC BEH 300, C18, 1.7 µm, 2.1×150 mm; Waters Corp., Milford, MA, USA); the MS/MS spectrum was recorded with an HPLC/ESI-MS/MS (HPLC Waters Alliance 2695 with a triple quadrupole mass spectrometer Waters 4 Micro) using a RP column (Jupiter Phenomenex 5 µm C18 90 Å

mm).

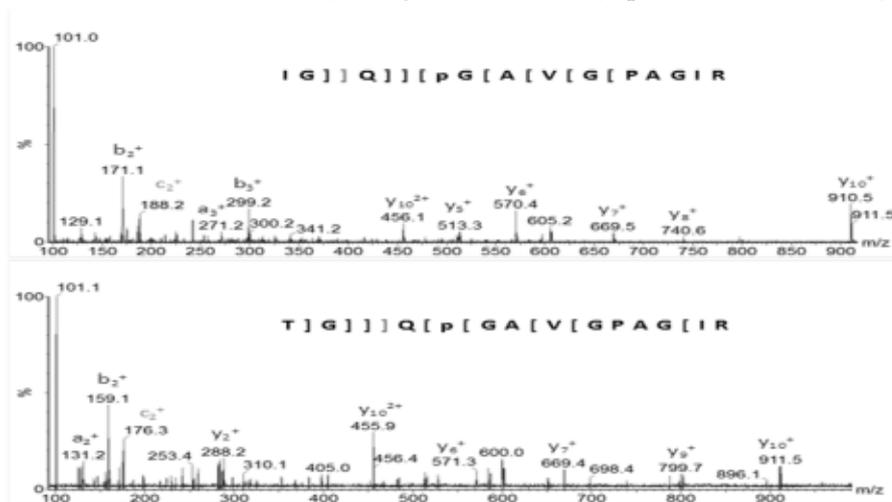


Fig. 1: MS/MS spectra of the marker peptide for beef (upper) and pork (lower) meat

The LC-HRMS study was accomplished using an HPLC DIONEX Ultimate3000 coupled with an LTQ Orbitrap XL (both from Thermo Fisher Scientific, Waltham, Massachusetts, USA) using an RP column (Jupiter Phenomenex C18 4µm, Proteo 90Å 150*0.30 mm). In all the experiments, the chromatographic runs were performed with similar gradient elutions with water and Acetonitrile (both with 0.1% formic acid)

Results & Discussion

The protein extraction and digestion protocol presented above was designed in order to set up the method and to identify the proper marker peptides.

After Full Scan and MS/MS analyses, two peptides were selected as the most discriminant markers between beef and pork meat on Bolognese sauce; their exact amino acid sequence was determined (IGQpGAVGPAGIR for beef and TGQpGAVGPAGIR for pork, refer to figure 1)

and their quantification was performed with MRM mode and SIR mode. The results obtained are fully comparable, so for the first time it was proved that a Single Ion Recording mode could be applied to assess meat authenticity.

A calibration curve with six different Bolognese sauces with a known meat composition, ranging from 100% beef/0% pork to 0% beef/100% pork was analysed and a good linear correlation ($r^2 > 0.99$) was found for both the meats.

To check the accuracy of the method, three blind samples (at unknown meat composition) were analysed and the results obtained are presented in the table below:

Unknown sample	Real beef %	Real pork %	Calculated beef %	Calculated pork %
7	35	65	26.1±0.1 (75%)	67.0±2.5 (103%)
8	75	25	70.4±7.9 (94%)	26.6±7.2 (106%)
9	98	2	80.50±0.03 (82%)	1.7±NA (85%)

Tab. 1: Results of the absolute quantification of beef and pork meat in the three blind samples

The results highlight that the method is able to quantify also very low pork percentages (2%).

Final Outcomes

The present work represents a step in advance in tandem mass spectrometry applications to detect and quantify beef and pork meat in complex and highly processed food matrices. The method, here successfully developed and validated, gave results particularly satisfactory in consideration of the total absence, to date, of analytical strategies able to quantify different meat species in these kinds of complex food matrices.

Furthermore, it could be actually implemented in food industries to check the meat composition of the final product but also to protect itself through the control of the raw materials or intermediate products supplied by other producers. Finally, it involves the use of relatively low cost instruments and, once set up, it does not require a highly specialized technicians, having also the potential flexibility to be extended to other meat species, on the basis of industry needs.

¹Sentandreu, M. Ā. n., & Sentandreu, E. (2014). Authenticity of meat products: Tools against fraud. *Food Research International*, 60(0), 19-29.

²Ballin, N.Z. (2010). Authentication of meat and meat products. *Meat Science*, 86, 577-587.

³Prandi B., Lambertini F., Faccini A., Suman M., Leporati A., Tedeschi T., Sforza S. (2017). Mass spectrometry quantification of beef and pork meat in highly processed food: application on Bolognese sauce. *Food Control*, 74, 61-69.

UHPLC/ESI-MS/MS detection of technical enzymes in wheat flour

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Background

The wide spectrum of food products that are wheat based can be ascribed to the rheological properties of wheat flour, and more specifically to the gluten network that is formed when flour and water are mixed to form dough. Besides gluten, other protein classes are implied in determining wheat product quality, among which the enzymatic pattern of the kernel. When endogenous enzymes of the kernel are not enough to reach the desired rheological properties, exogenous enzymes can be added as technological adjuvant.¹ These enzymes belong to different classes and they act in a different way, and each enzyme has its own industrial applications. They are generally recognized as safe (GRAS) and they are denatured during baking, thus they do not require to appear on the label. These enzymes are used in very low concentrations (usually less than 200 ppm), thus sensitive analytical methods are required to detect their presence. Since it is a recently emerged issue, few or none (depending on the enzyme) analytical methods are available. Most of them rely on a typical proteomic approach using electrophoresis, tryptic digestion and mass spectrometry analysis for protein identification and liquid chromatography coupled to mass spectrometry for its quantification.

Case Study: marker peptides identification for technical enzymes

Thirteen different enzymes were analysed from AB, Danisco, DSM and Novozymes. Among the enzymes that act on the carbohydrate fraction, we considered Bakezyme 800 (a glucosidase), Fungamyl Ultra BG (a α -amylase), Bakezyme MAM 5000 L and Novamyl (two maltogenic amylases). Also enzymes acting on non-starch polysaccharides were considered, such as Pentopan Mono Conc BG and Grindamyl H490, two xylanases. The last class of carbohydrate related enzymes is glucose oxidase (Grindamyl S 758 and Gluzyme Mono Conc BG). We analysed also three different proteases (Neutrase 1.5MG, Bakezyme PPU 95000, Grindamyl PR43 and Veron HPP). Despite the lipid fraction of flours is very poor, also an enzyme acting at the lipid level was taken into account, a glycolipase (Powerbake 4090).

Novelty statement

Up to date, only one analytical method² was published for the detection of one technical enzyme preparation (Fungamyl Super AX). It is a commercially available Novozymes blend of two enzymes: Pentopan 500 (GH11 xylanase) and Fungamyl (alpha-amylase).

Experimental

The sequence of the enzymes present in the commercial formulation was determined using SDS-PAGE followed by in gel digestion and HRMS analysis. Briefly, ten milligram of each sample were extracted in 1 ml of extraction buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl

sulphate, 350 mM dithiothreitol), incubating for 1 hour at room temperature. Samples were centrifuged at 11337g for 10 min at room temperature and supernatants were loaded in the electrophoretic gel. Protein bands were excised from the gel, detained and submitted to in gel tryptic digestion. Peptides were analysed with μ HPLC coupled to LTQ-OrbiTRAP and protein were identified with Proteome Discover software. Proteins were then extracted in denaturing conditions (ammonium bicarbonate 100 mmol L⁻¹, urea 4 mol L⁻¹ and dithiothreitol 5 mmol L⁻¹). After alkylation with iodoacetamide (12.5 mmol L⁻¹), proteins were submitted to tryptic digestion. Peptides generated were analysed with reverse phase UHPLC/ESI-MS/MS and identified by means of free on line softwares (FindPept and ProteomicsToolkit). Specificity of identified peptides was checked by Basic Local Alignment Search Tool (BLAST) in Uniprot database. Selected reaction monitoring (SRM) method was developed optimizing the mass spectrometer parameters.

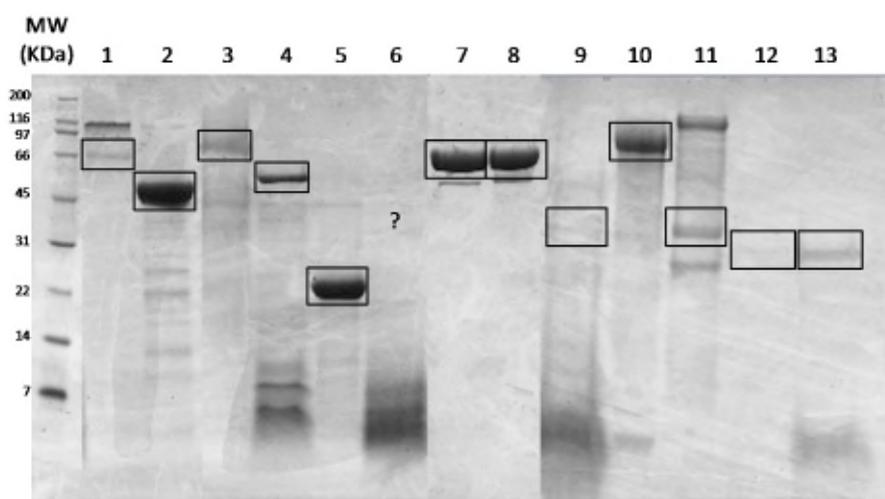


Fig. 1. Gel electrophoresis of the enzyme preparations under investigation. MW- molecular weight marker, 1-Bakezyme AG800, 2-Fungamyl Ultra BG, 3-Grindamyl S 758, 4-Neutrase 1.5MG, 5-Pentopan Mono Conc. BG, 6-Powerbake 4090, 7-Bakezyme MAM 5000 L, 8-Novamyl, 9-Bakezyme PPU 95000, 10-Gluzyme Mono Conc. BG, 11-Grindamyl H 490, 12-Grindamyl PR 43, 13-Veron HPP.

Results & Discussion

This study identified unique marker peptides for thirteen commercial enzymes used in bakery. SRM methods were developed to detect the marker peptides in flour samples at low concentrations. Blank flours were analysed to ensure the absence of the marker peptides as endogenous compound already present in flour. Anyway, the limit of detection of the method needs to be improved, due to the very low added concentration of these enzymes (50-200 ppm of enzyme preparation, corresponding to about 2.5-10 ppm of pure enzyme).

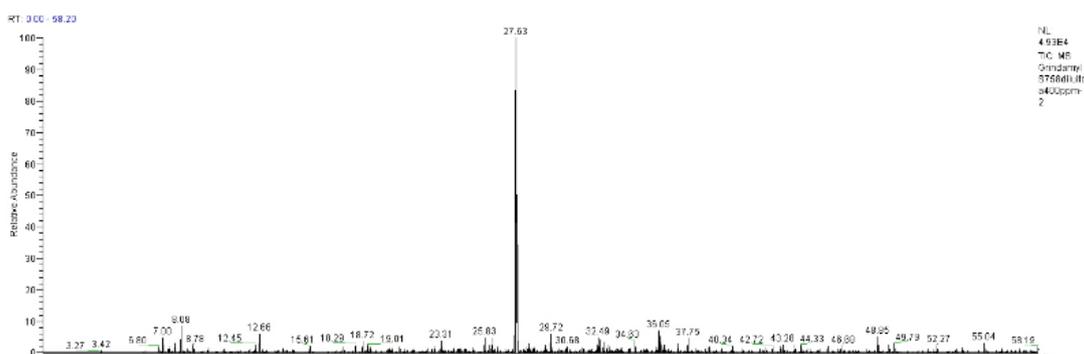


Fig. 2. SRM detection of Grindamyl S758 (a glucose oxidase) at 400 ppm in water.

Final Outcomes

The results suggest that UHPLC/ESI-MS/MS is suitable to identify commercial enzymes used in bakery as technological adjuvants, but improvements are needed to lower the limit of detection of the method. Further experiments will be performed to achieve a correct quantification of these enzymes using appropriate calibration curves.

¹ Melim Miguel, A. S., Martins-Meyer, T. S., da Costa Figueiredo, E. V., Paulo Lobo, B. W., Dellamora-Ortiz, G. M. (2013) **Enzymes in Bakery: Current and Future Trends.** *Food Industry*, Chapter 14, 287-321.

² Uhr, L., Wieland, P., Homann, T., Huschek, G., Rawel, H. M. (2016) **Identification and LC-MS/MS-based analyses of technical enzymes in wheat flour and baked products.** *European Food Research and Technology*, 242(2), 247–257.

Intelligent Quality Assurance – Non-targeted analysis to determine biomarkers associated with meat discolouration

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Background

Consumers associate the colour of meat as a measure of quality¹ even though it has been demonstrated that the appearance of meat colour does not always determine eating quality². The colour of beef is controlled predominantly by the presence and oxidation state of myoglobin which can be influenced by several factors including pH, atmosphere and temperature. Undesired discolouration of meat was occasionally observed. Initial investigations by the ABP Food Group have not been able to identify the cause for this discolouration. Therefore, a nontargeted analysis was proposed to determine metabolite differences between meat samples with varying discolouration. Determination of the chemical processes that are occurring during meat packing will assist in the identification of the root cause of the discolouration.

Case Study: NMR and LC-HRMS analyses to determine biomarkers associated with meat discolouration

Aim of this work is try to use the profiling techniques of ¹H Nuclear Magnetic Resonance (NMR) spectroscopy and liquid chromatography high resolution mass spectrometry (LC-HR-MS), in order to profile samples of meat that exhibit discolouration and to identify significant differences when compared to ‘normal’ samples with a desirable appearance.

Novelty statement

This study purposes a robust approach able to find the analytes responsible of meat discolouration: the results obtained with two confirmatory techniques (NMR and HRMS) are merged in order to identify an higher number of markers.

Experimental

Samples of fresh, raw beef showing degrees of discolouration were collected from ABP Food Group.

To prepare samples for chemical analysis, 5 g (± 10 mg) of samples were cut from each steak.

Water was added to each steak sample at a ratio of 2 mL per gram of steak in each sample prior to homogenisation over ice by turrax. The samples were homogenised over two 30-second periods following which the samples were centrifuged ($2045 \times g$, 20 °C, 10 minutes) and the supernatant decanted.

The following stages were completed twice: 2 mL of ice-cold acetone was added to 1 mL of the supernatant solution over ice. The precipitated samples were centrifuged and the supernatant decanted. The remaining steps undertaken were specific for the analysis type and are detailed below

- *NMR analysis*: supernatants were transferred to a glass vials and placed under a stream of nitrogen gas until the solvent had been reduced by ~75%. Samples were frozen and lyophilised to dryness. All lyophilised samples were reconstituted in 800 μ L of phosphate buffer solution (100 mM pH = 7.0) made up in ²H₂O containing 1 mM TSP.

540 μL of the reconstituted samples and 60 μL of sodium azide solution (final concentration 1 mM, made up in $^2\text{H}_2\text{O}$) were added to labelled 5 mm NMR tubes and samples were analysed in a random order.

All ^1H NMR experiments were carried out at 300 K using a Bruker Avance 500 MHz NMR spectrometer equipped with a TCI cryoprobe. Data acquisition was performed with the use of Topspin v 1.3 (Bruker, Germany). Spectra were acquired at a central frequency of 500.1323546 MHz

- *LC-HRMS analysis*: supernatants were diluted 10 fold by adding 150 μl of sample to 1350 μl of purified water (18.2 Ω) before analysis by Liquid Chromatography –High Resolution Mass Spectrometry. Samples were analysed in a random order. Liquid Chromatography was undertaken on an Accela system and the mass spectrometer was an Exactive Orbitrap MS, both from Thermo Scientific. The analytical column used was an ACE AQ 150 mm x 3 mm, 300 \AA . Mobile phase A was 0.1% formic acid in HPLC water, mobile phase B was 0.1% formic acid in acetonitrile. A linear gradient elution was applied over 20 minutes from 100% MPA to 100% MPB. The LC flow rate was 0.4 mL min⁻¹ and the column temperature was 25°C. Sample injection volume was 10 μL .

Results & Discussion

A typical ^1H NMR spectrum acquired from a meat sample is shown in Figure 1.

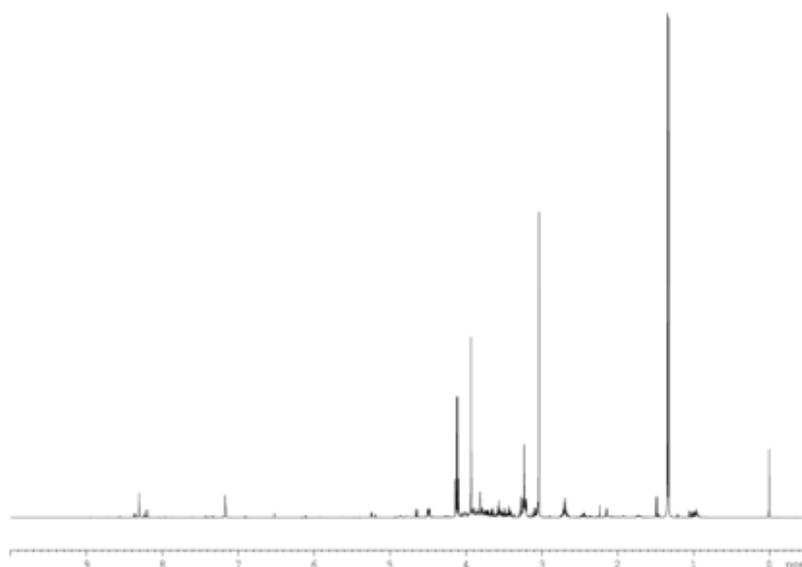


Fig.1: 500 MHz ^1H NMR spectrum taken from a discoloured meat extract

The instrument highlighted 958 features in the NMR spectra acquired from the meat samples. Thirty two of the features were shown to be significantly different ($p > 0.001$) between thin cut steaks which were and were not discoloured. Tentative assignment of these features determined that the concentration of the following compounds was significantly different between normal and discoloured thin cut steaks: phenylalanine, tyramine, acetyl carnitine, an unidentified phosphorylated carbohydrate, lysine, valine and leucine.

Three features could not be tentatively assigned.

For HPLC-HRMS data elaboration, the results obtained from the discoloured beef steaks against normal steaks were compared.

After chemometric evaluation, eight potential metabolites were observed with a significant higher area ($p < 0.01$) in the discoloured samples. Figure 2 shows an example of the response plot obtained for these metabolites and Table 1 list their mass information, p values and possible identification:

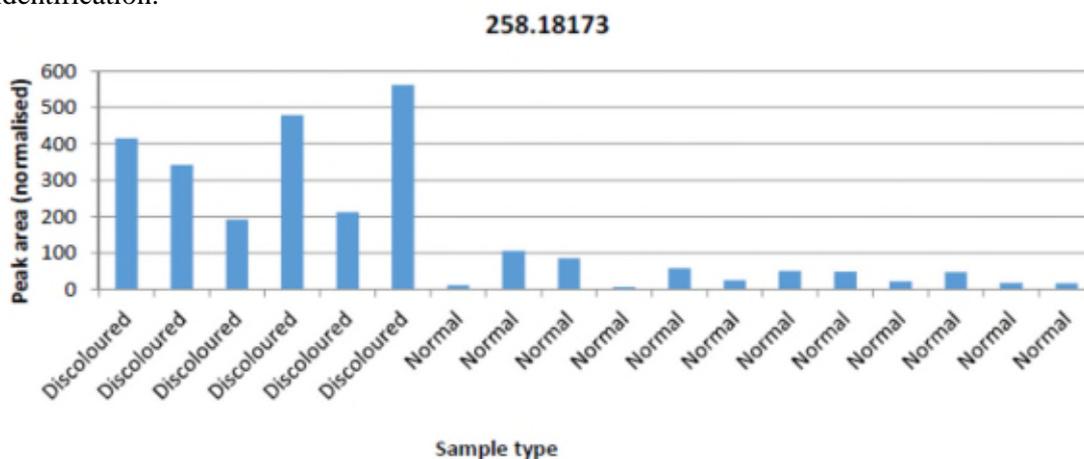


Fig. 2: m/z 258.18173 response across discoloured and normal (control) steaks

Mass (m/z)	p-value: discoloured vs normal	Tentative identification	Positive or negative ion
258.18173	0.00000579	Ile-Lys dipeptide	Negative ion
295.09271	0.000119	Asp-Tyr dipeptide	Negative ion
311.10649	0.000733	Methionyl-Tyrosine	Negative ion
303.09563	0.00893	Gly-Asp-Asn tripeptide	Negative ion
350.13487	0.0000546	Gly-Phe-Glu tripeptide	Negative ion
320.16083	0.0000109	Gly-Val-Phe tripeptide	Negative ion
315.84020	0.0014	No identification	Positive ion
305.52407	0.0004	No identification	Positive ion

Tab. 1: List of target metabolites

Final Outcomes

A number of analytes were identified that are significantly different when comparing discoloured against normal steaks.

The analysis of data by ^1H NMR spectroscopy identified several differences in the concentration of several metabolites and the majority of these compounds were amino acids (phenylalanine, leucine, lysine, valine) or breakdown products of amino acids (tyramine).

In the case of analysis of steaks using LC-HRMS, eight features were identified that were significantly different between normal and discoloured steaks. Six of these were tentatively assigned and the majority of these were tri or di peptides.

All metabolites identified by either NMR or LC-HRMS were breakdown products of proteins but it was not possible using the data collected to identify which proteins were degrading.

The colour of beef is controlled by the oxidation and reduction of the protein myoglobin. A brief literature review determined that meat digestibility by external enzymes is increased at elevated temperatures, leading to the formation of peptides and amino acids. It was observed that a temperature of 70°C was sufficient to denature proteins, and temperatures of 65°C have been demonstrated to denature myoglobin³ leading to discolouration.

It was noted on the preliminary site visit that the machinery used for production of retail vacuum skin packs, produced packages that contained a significant amount of heat. It is therefore hypothesised that the discolouration is due to the denaturation of the protein myoglobin. If this hypothesis is correct, control of the temperature of the meat is paramount to removal of the

discolouration effect.

To confirm the hypothesis, the number of units produced with discolouration over a given time period should be monitored and compared to the same time period with the control measure in place.

¹D. J. Troy, J. P. Kerry, Consumer perception and the role of science in the meat industry, *Meat science* 86, 214-226 (2010)

² K. G. Grunert, L. Bredahl, K. Brunsø, Consumer perception of meat quality and implications for product development in the meat sector—a review. *Meat science* 66, 259-272 (2004)

³ M. C. Hunt, O. Sørheim, E. Slinde, Color and Heat Denaturation of Myoglobin Forms in Ground Beef., *Journal of Food Science* 64, 847-851 (1999)

Case study to detect the fraudulent adulteration of vinegar and balsamic vinegar using Isotope Ratio Mass Spectrometry Analysis

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Background

By definition (EC Regulations 479/2008, Annex IV, sections 1 and 17) wine vinegar is the product obtained from the acetous fermentation of wine, which is in turn defined as a product obtained exclusively from the alcoholic fermentation of fresh grapes, whether crushed or not, or from grape must. According to this definition, wine vinegar cannot contain synthetic acetic acids (e.g. from petroleum derivatives or pyrolysis of wood) or acetic acid from the fermentation of nongrape sugars (e.g., from beet or cane). Moreover, wine and wine vinegar cannot be produced from dried grapes diluted with water; therefore, the so-called “raisin vinegar”, commonly produced in some Mediterranean countries by fermenting dried grapes and rehydrating with tap water, cannot be considered wine vinegar. This also applies to “aceto balsamico di Modena IGP” (ABM), a PGI (Protected Geographical Indication) vinegar now renowned throughout the world, obtained from cooked and/or concentrated grape must (at least 20% of the volume), with the addition of at least 10% of wine vinegar and a maximum 2% of caramel for color stability (EU Reg. 583/2009). The geographical origin of ABM ingredients is not specified.

Since 1991 the addition of water and exogenous sugars to grape must and wine, has been detected by analyzing the isotopic ratios of hydrogen ($^2\text{H}/^1\text{H}$) and carbon ($^{13}\text{C}/^{12}\text{C}$) in ethanol and of oxygen ($^{18}\text{O}/^{16}\text{O}$) in water. OIV (International Organization of Vine and Wine) methods are currently officially adopted to detect these kind of frauds (OIV-MA-AS311-05 for site-specific analysis of the $^2\text{H}/^1\text{H}$ ratio using ^2H -site-specific natural isotope fractionation NMR, ^2H -SNIF-NMR; OIV-MA-AS312-06 for analysis of the $^{13}\text{C}/^{12}\text{C}$ ratio, as $\delta^{13}\text{C}$ ‰, using isotope ratio mass spectrometry, IRMS; OIV-MA-AS2-12 for analysis of the $^{18}\text{O}/^{16}\text{O}$ ratio, as $\delta^{18}\text{O}$ ‰, using IRMS). The fraud is detected by comparison of the isotope data of the samples against an official databank (EU Regulations 2347 and 2348/91) set up since 1991 by the European Union for all wine-producing countries within its territory. The isotope data bank makes available reference data on a yearly basis, thus allowing legal limits to be defined on the basis of isotopic data for each country, each subarea (e.g., region), and each protected denomination (PDO-IGP), as well as general limits when origin and year of production are not declared.

Recently, isotopic methods have been recognized by the European Committee for Standardization (CEN) and in part by OIV as a means of detecting the presence of exogenous acetic acid and tap water in wine vinegar (EN 16466-1 method for determination of $^2\text{H}/^1\text{H}$ in the methyl site of acetic acid using ^2H -SNIF-NMR, EN 16466-2; OIV 510/2013 for analysis of $^{13}\text{C}/^{12}\text{C}$ in acetic acid using IRMS; EN 16466-3 and OIV 511/2013 for analysis of $^{18}\text{O}/^{16}\text{O}$ in water using IRMS).

Case study (More details in Camin et al., 2013, Food Control and Perini et al., 2014, Journal of Agricultural and Food Chemistry)

The aim of this study was first of all to prove experimentally that raw vinegar “memorises” the isotopic composition of the relevant wine without significant differences and therefore that the

official isotopic wine databank can be taken as reference also for vinegar. Moreover, the applicability of $\delta^{18}\text{O}$ analysis for the detection of fraudulent production of wine vinegar from rehydrated dried grapes was investigated.

Furthermore, we investigated whether official isotopic methods recognized by OIV and CEN for must and wine vinegar can be used to analyze ABM, despite the addition of ingredients different from grape products (i.e. caramel) and the complex production process.

Novelty statement

For the first time this study presents an attempt to use an official method for wine adulteration also for vinegar and ABM adulteration.

Experimental

For all the details see Camin et al., 2013, Food Control and Perini et al., 2014, Journal of Agricultural and Food Chemistry.

Results and Discussion

This study demonstrates experimentally that acetic fermentation of wine does not affect the $\delta^{18}\text{O}$ values of wine vegetal water. This means that the official wine databank and $\delta^{18}\text{O}$ analysis of water, officially used to detect the watering of wine and rehydration of concentrated fruit juice, can also be applied to wine vinegar to determine this kind of fraud.

On the basis of extensive data in the official wine databank, lower limits were fixed for the $\delta^{18}\text{O}$ values of water contained in raw wine vinegar (-2‰) and in commercial diluted vinegar (-5‰). Such limits have proved to be usable for assessing the authenticity of vinegars produced in Italy and in Mediterranean. For other origins these limits must be confirmed on the basis of the corresponding isotopic wine databank, whereas for specific cases (declaration of origin from a narrow area) the specific limits of the databank can be taken into account.

As regards in the specific ABM, the 'impurities' found in the extraction solution do not affect the $^{13}\text{C}/^{12}\text{C}$ of acetic acid and the $^2\text{H}/^1\text{H}$ values of acetic acid. Furthermore, the repeatability and reproducibility of the methods are comparable in wine vinegar and ABM and generally lower than those quoted in the official methods. This means that the validation parameters quoted in the official methods can also be applied to the ingredients of ABM. In addition, no changes in the isotopic values from wine to vinegar and to ABM, and from the original must to the ABM must were found,

providing experimental evidence that reference data from wine databanks can also be used to evaluate the authenticity of vinegar and ABM.

Conclusions and final outcomes

These studies experimentally demonstrated that EU official methods used to detect the fraudulent addition of water and exogenous sugars to grape must and wine can also be applied to wine vinegar and ABM. Moreover, reference data from official EU wine databanks can also be used to evaluate the authenticity of vinegar and ABM.

For the further validation, FEM is organizing an interlaboratory comparison of the method with other isotopic analysis well-experienced European laboratories (e.g. Eurofins Scientific - France, Laboratorio Arbitral Agroalimentario - Ministerio de Agricultura – Spain, The method will be tested for both the matrices wine vinegar and ABM, Institut für Lebensmittelchemie, Landesuntersuchungsamt – Germany)

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Case study: protect PDO, PGI and TSG cheeses against mislabelling frauds using Isotope Ratio Mass Spectrometry Analysis

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Background

European laws EC N. 510/2006 and 1151/2012 require protection against the mislabelling of foods with Protected Geographical Indications (PGIs), Protected Designations of Origin (PDOs) and Traditional Specialities Guarantee (TSG). In first place in Italy in terms of sales of these kind of products, are the two PDO hard cheeses Grana Padano and Parmigiano Reggiano. Several other hard cheeses without any certified origin designation are available on the market, however, with a wholesale price which is less than half that of the two PDO cheeses. Especially when the cheese is sold in grated or shredded form, easy profits can be made by dishonest producers by fraudulently labelling common non-PDO cheeses with the two most famous designations. This is why it is desirable to develop objective and effective methods capable of identifying the origin of the cheese used to prepare pre-packed grated or shredded products, when the usual check on the original branded PDO logo on the rind is not possible.

A recent paper showed that by combining the stable isotope ratios of H, C, N and S and the elemental profile of cheese it was possible to trace the origin of seven types of European hard cheeses and specifically to identify PDO Parmigiano Reggiano cheese from non-PDO imitations. Moreover, isotopic analysis was officially adopted in 2011 as a reference method for verifying the authenticity of PDO Grana Padano cheese (EU Reg 584/2011). The methods for analysing H, C, N and S stable isotope ratios using Isotope Ratio Mass Spectrometry (IRMS) and the elemental profile using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) have been described in several papers but they have not been backed up by collaborative studies, as required nowadays by all official method standardisation bodies. This is necessary to make validated and officially recognised methods [e.g. by European Committee for Standardisation (CEN), AOAC International] available in commercial disputes and legal debates.

Case study

A collaborative study was organized and coordinated by the Fondazione Edmund Mach and sponsored by the Consortium for the Protection of the Grana Padano PDO to validate the use of isotopic and elemental composition methods to detect its mislabeling. The study was organised and performed according to the International Union of Pure and Applied Chemistry (IUPAC) protocol and International Organisation for Standardisation (ISO, Geneva, Switzerland) Standards 5725/2004 and 13528/2005.

The isotopic ratios of H, C, N and S were determined, as well as the content of the 13 elements Li, Na, Mn, Fe, Cu, Se, Rb, Sr, Mo, Ba, Re, Bi, U. These parameters were found to be the most effective in tracing the origin of both Grana Padano and Parmigiano Reggiano according to a previous study and the analytical methods proposed guaranteed repeatable results, at least on an intra-laboratory scale.

Twenty laboratories were initially involved in the collaborative study, but only 13 of them completed the entire procedure, sending in the results. Three laboratories performed both isotope and elemental analysis, seven only isotopic analysis, and three only elemental analysis.

Novelty Statement

This study introduces the use of the isotopic ratio evaluation for the detection of mislabeling in Grana Padano PDO.

Experimental

For all the details see Camin et al., 2015, Rapid Communications in Mass Spectrometry.

Results and Discussion

Stable isotope ratio analysis

Ten laboratories presented isotope ratio results and none of them was eliminated as a technical outlier, although some of them did not apply the exact protocol. This means that the use of other extraction devices (Soxhlet extractor instead of the matching Ultraturrax centrifuge) or even solvents (water/dichloromethane, 2:1, and acetone instead of petroleum ether/diethyl ether, 2:1) does not have a significant impact on the variability of the isotopic ratios and can be used as an alternative.

The minimum of five laboratories supplying valid results required in the IUPAC harmonised protocol for collaborative studies on complex methods was satisfied for all the isotope ratios. The data, especially for the $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values, cover almost all the range of variability found for European hard cheeses. The standard deviations of repeatability (sr) and reproducibility (sR) for each sample were calculated considering only the valid results of the blind duplicates according to the ISO Standard 5725 and the IUPAC protocol. In general, the sr and sR values obtained for all materials are comparable, despite the fact that some samples were fresh and others freeze-dried. It follows that the freeze-drying process does not have any significant effect on isotopic variability.

For the $\delta^{13}\text{C}$ values the average standard deviation of repeatability (sr) was 0.1 ‰ and that of the reproducibility (sR) was 0.2 ‰. For all the samples, sr and sR were comparable with or even lower than those previously observed for wine ethanol (OIV 312-06), honey bulk and protein (AOAC 998.12), and fruit juice sugar and pulp (ENV 12410, ENV 13070), for which stable isotope analysis has been validated since the 1990s. For the $\delta^{15}\text{N}$, $\delta^2\text{H}$ and $\delta^{34}\text{S}$ values, the average standard deviations of repeatability (sr) were 0.1‰, 2‰ and 0.4‰, respectively, whereas those of reproducibility (sR) were 0.3‰, 3‰ and 0.6‰, respectively. For these elements, validated methods do not yet exist. However, the sr and sR values are comparable with those found in the literature for several food matrices, including dairy products.

The results of two samples prepared by mixing different cheeses, were compared with the expected calculated values. The differences between the measured and the expected values for the $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^2\text{H}$ and $\delta^{34}\text{S}$ values were always below the repeatability limits (expressed as $2.8 \times \text{sr}$, according to the IUPAC protocol).

Elemental analysis

Six laboratories presented their results for elemental analysis and none of them was eliminated as a technical outlier, although some of them did not apply the exact digestion protocol. In general, the elemental concentration ranges of the samples in this study were consistent with those of commercial hard cheeses. The content of Li, Na, Mn, Fe, Cu, Se, Rb, Sr, Mo, Ba and Bi was found to be over the detection limit (DL) in at least one sample in all six laboratories, only in 5 laboratories for U, and in 3 for Re. Due to instrumental method limitations, results for Re were not provided by three laboratories. Thus, for this last element, always present in these cheeses at levels below 1 µg/kg, no statistical processing was possible.

The repeatability relative standard deviation (RSDr) and reproducibility relative standard deviation (RSDR), calculated considering only the valid data were:

- Li: RSDr 10% and RSDR 28%, respectively, ranging between 3 and 28% for r and between 20 and 41% for R;
- Na: RSDr 2% and RSDR 13%, with a min-max range of 1–3% and 8–15%, respectively;
- Mn: RSDr 10% (min-max: 3–19%) and 13% (10–17), respectively;
- Fe: RSDr 11% and RSDR 23%, respectively, with RSDr ranging from 4 and 30% and RSDR from 15 to 44%;
- for Cu: RSDr was 7%, ranging between 1 and 19%, while the average RSDR was 19%, ranging between 11 and 28%;
- Se: RSDr varied from 2 to 22%, with an average of 10%, while RSDR varied from 9 to 30 %, with an average of 19%;
- Rb: RSDr was 3%, ranging between 1 and 5%, while RSDR was 12%, ranging between 10 and 14%;
- Sr: RSDr was 4% (range 1 - 9%), while the average RSDR was 12% (range 10 - 16%);
- Mo: RSDr and RSDR values were 5% (range 2 - 10%) and 17% (range 11 - 23%);
- Ba: the average RSDr was 3%, ranging from 1 to 7%, while the average and RSDR was 9%, ranging from 4 to 19%;
- Bi: RSDr varied between 3 and 6% and the RSDR between 12 and 13%;
- U: the RSDr ranged between 5 and 10% and the RSDR between 13 and 26%;
- Re: statistical processing was not possible.

The use of fresh cheeses (instead of lyophilised cheeses) requires particular attention during the preparation of samples that must be carefully homogenised before mineralisation. This seems particularly important for Rb and Cu determinations that on fresh cheeses generally showed a worse intra-lab repeatability than on lyophilised samples.

The RSDr and RSDR results obtained were consistent with the data reported by FDA (Food and Drug Administration, Elemental Analysis Manual, section 4.4. Available: www.fda.gov).

The results of the two samples prepared by mixing different cheeses, were compared with the expected calculated values. Generally the differences between the results and the expected values were not statistically significant (t-test, $p > 0.05$), except for Mn, Ba and Re for one sample ($p = 0.043$, 0.003 and 0.031 , respectively).

Conclusions and final outcomes

An international collaborative study was organised according to the IUPAC protocol and ISO Standards 5725/2004 and 13528/2005 to determine the performance and validation data of methods for the isotopic and elemental analysis of cheese. The results for stable isotope ratios are comparable with or even better than those found in official methods for other food matrices and in the literature. The values for the elemental composition are consistent, in regard to precision, with methods reported by FDA and in the literature for cheese. For Re it was not possible to determine validation data.

For isotopic analysis the differences between the results and the expected values were good and for mineral elements they were not statistically significant, except for Mn, Ba and Re in one sample. The use of different extraction devices or even solvents does not have a significant impact on the variability of the isotopic ratios. Similarly, different lipid extraction methods and mineralisation procedures do not have a significant impact on the variability of the elemental data. The performance data obtained in this study were submitted to the Italian standardisation agency UNI (Italian Organization for Standardization) to obtain an official recognition of the method.

This last step is necessary when this method is used to verify the mislabelling of PDO cheese in commercial disputes or legal debates. At the moment the method resulted validated by UNI and it is in the final acceptance stage.

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Case study to detect the fraudulent adulteration of Italian citrus juices and tomato passata using Isotope Ratio Mass Spectrometry Analysis

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Background

Isotopic methods based on the determination of isotope ratios by Isotope Ratio Mass Spectrometry (IRMS) and Site-Specific Natural Isotope Fractionation-Nuclear Magnetic Resonance (SNIF-NMR) are possibly one of the most efficient techniques to date to detect whether a fruit or a vegetable juice was added with water or sugars without declaring it. These methods are now well established and have been collaboratively tested both in Europe (European Committee for Normalisation – CEN: ENV 12140, ENV 12141, ENV 13070 methods) and internationally (Association of Official Analytical Chemists: AOAC 995.17 and AOAC 2004.01 methods).

Beside the methods, it is important to define threshold values to refer to, when evaluating if a fruit juice or a tomato passata is genuine or not. This is why the European Fruit Juice Association (AIJN) of producers have co-operated to set some reference guidelines, known as the European Quality Control System (EQCS), by which the quality and authenticity of fruit juices can be judged.

The most commonly practised types of fraud concerning fruit juices are the addition of sugar or water as well as the substitution of the named fruit with a cheaper variety (United States Pharmacopeia – UPS, data December 2013). In this context in 2012 the European Parliament enacted a directive (2012/12/EU) defining that a fruit juice is “The fermentable but unfermented product obtained from the edible part of fruit which is sound and ripe, fresh or preserved by chilling or freezing of one or more kinds mixed together having the characteristic colour, flavour and taste typical of the juice of the fruit from which it comes” (Annex 1). Furthermore, it establishes that if a fruit juice is obtained by the watering down of a concentrate it have to be clearly indicated on the label as “fruit juice from concentrate” and finally that sugar addition is no longer allowed in fruit juices.

The potential of stable isotope techniques to detect fruit juice adulteration could considerably improve by carrying out the determination on several components of the same product and investigating their intermolecular isotopic correlation. It has been shown that specific correlations exist between the ^{13}C value of different metabolites of a fruit (e.g. organic acids, sugars), particularly if they arise from the same metabolic pathway, and that any deviation from the expected correlation is indicative of the addition of at least one of these compounds from an exogenous source.

To what concern the tomato passata, according to Italian law (D.M. 23rd September 2005), it must derive only from fresh tomatoes. The natural passata have to be obtained by grinding the tomatoes and normally centering them through a minimal evaporation process with partial removal of water so that the optical refractometric residue lies between 5 and 12 Brix degrees, with a tolerance of 3%, net of the added salt. It is not permitted to reconstitute passata using tomato paste (with a Brix degree over 12°) with the addition of water. The same decree establishes that in order to detect this fraud, analysis of the isotopic ratio of oxygen ($^{18}\text{O}/^{16}\text{O}$, expressed as $\delta^{18}\text{O}$) should be used as has taken place for many years for fruit juices (Reg. EC 822/97, ENV 12141).

However, Italian law does not give any information on the acceptable range of $\delta^{18}\text{O}_{\text{water}}$ values indicating whether tomato passata is authentic or not.

Few authors in the international scientific literature have studied the ranges of $\delta^{18}\text{O}$ values in the vegetal water of tomatoes and derivatives and nobody has verified the potential of this parameter in detecting the fraudulent addition of water during the production of tomato passata.

Case study

Citrus juices (More details in Bontempo et al., 2014, Journal of Mass Spectrometry)

The aim of this study was to determine, for the first time in an extensive way (more than 500 samples), the characteristic ranges of variability for stable isotope ratios determined in several fractions (water, ethanol produced from the fermentation of juice, pulp, sugars and acids) of genuine Italian citrus juices, and also to investigate the relationships between metabolites. The final scope was to verify the compliance of these ranges with the limits proposed by AIJN Code of Practice and by the literature.

Tomato passata (More details in Bontempo et al., 2014, Food Control)

The aim of the work was to establish a characteristic range of $\delta^{18}\text{O}$ values for the natural Italian tomato passata using a very extensive database, including many harvest years and different origins, in order to establish a threshold limit that could eventually become official and be adopted to implement the Ministerial Decree (D.M. 23rd September 2005). For this reason, due to the dual interpretation of the Ministerial Decree, there was verification on the one hand of the extent to which $\delta^{18}\text{O}_{\text{water}}$ is influenced by dilution passing from paste to 'false' passata and on the other hand whether it is possible to differentiate watered down passata, previously concentrated up to 11.9 Brix, from products obtained using watered down tomato paste (with original Brix higher than 12).

Novelty Statement

For citrus juices, for the first time the characteristic ranges of variability for stable isotope ratios determined in several fractions (water, ethanol produced from the fermentation of juice, pulp, sugars and acids) of genuine Italian citrus juices is determined.

For tomato passata, a characteristic range of $\delta^{18}\text{O}$ is evaluated in order to establish a threshold limit that potentially could become official.

Experimental

For all the details see Bontempo et al 2014, Journal of Mass Spectrometry and Bontempo et al. 2014, Food Control.

Results and Discussion

Citrus juices

In Bontempo et al. 2014, Journal of Mass Spectrometry, the mean, standard deviation, median, 25th and 75th percentile values of $\delta^{13}\text{C}_{\text{pulp}}$, $\delta^{18}\text{O}_{\text{water}}$, $\delta^{15}\text{N}_{\text{pulp}}$, $\delta^{13}\text{C}_{\text{ethanol}}$, $\delta^{18}\text{O}_{\text{pulp}}$, $(\text{D}/\text{H})_{\text{I}}$, $(\text{D}/\text{H})_{\text{II}}$ and R of Italian citrus juices are summarised for orange and lemon juices and for orange concentrates.

Regarding compliance with the limits proposed by the AIJN, though considering the uncertainty of the method, some $\delta^{18}\text{O}_{\text{water}}$ values in Italian citrus juices fell below the minimum threshold 0‰ for both lemon and orange juices, by up to -2‰. Specifically, values below -0.2‰ were found in 7% of orange and lemon juices collected in different years (2001, 2003, 2004, 2005, 2007, 2009, 2010 and 2011), mainly during January and February periods. In particular, only one orange juice collected in 2007 showed a value of -2.0‰, while the other samples were between -0.2 and -1‰. If the limit of 0‰ was strictly applied, these samples would be considered adulterated by the addition of water although certainly genuine.

The situation for $(D/H)_I$ in orange juices is different as roughly all the sample values fell within the 103 and 107 ppm interval prescribed by the AIJN, taking uncertainty of the method into account. It should be noted, however, that the Code of Practice states that ‘the lower limit of 103 ppm is only approached in American origins; Mediterranean juices show values higher than 105 ppm’, which means that more than 36% of juices would be outside the tolerance range and would be considered adulterated by beet sugar. As highlighted in the Code of Practice (‘orange juices showing $(D/H)_I$ below 103 ppm [...] are associated with $\delta^{13}C_{ethanol}$ values also very low—below -27‰ ’), the Italian samples also confirmed the tendency of the lower values (<105 ppm) to be associated with $\delta^{13}C_{ethanol}$ values between -26.5 and -29.2‰ , even though a statistically significant correlation between these two parameters was not found. The distribution of $\delta^{13}C_{sugars}$ values in Italian citrus samples ranged between -27.8 and -22.8‰ ; both outside the threshold values proposed by AIJN (-27‰ and -24‰). Although the AIJN remarks on the rarity of juices with values between -23.5 and -24.0‰ and taking uncertainty of the method into account, 10% of the samples had values between -24 and -23.5‰ , in 7% of juices values were higher than -23.5‰ , and in 3% of samples they were lower than -27‰ . In the case of $\delta^{13}C_{ethanol}$ values, if uncertainty of the method was considered (0.3‰), the AIJN upper limit (-25‰) was met while 5% of the samples fell outside the lower one (-28‰). Regarding $\delta^{13}C_{pulp}$, the lower value found in the Italian citrus juices (-28.3‰) was within the limit proposed by AIJN (-28‰), but not the higher one (-22.5‰ vs the AIJN -23.5‰), above which 1.4% of the samples fell although considering the 0.5‰ uncertainty. Furthermore, the difference between $\delta^{13}C_{pulp}$ and $\delta^{13}C_{sugars}$ was between -3.3 and $+1.5\text{‰}$, which is not totally in agreement with the AIJN directive ($-1/+0.5\text{‰}$) although considering the pooled uncertainty (0.7‰). Regarding $\delta^{13}C_{acids}$, even though determined on a limited number of samples, taking into account the uncertainty of the method, only one sample was outside the lower AIJN threshold limit (-25.5‰) while the higher one (-22.5‰) was met. The differences between $\delta^{13}C_{acids}$ and $\delta^{13}C_{sugars}$ ranged between -0.6 and $+1.5\text{‰}$, and therefore, taking uncertainty of the method into account, the higher limit established by the AIJN ($+1$ to $+2\text{‰}$) was met, but not the lower one (20% of the samples fell below $+0.2\text{‰}$). In the case of $\delta^{13}C$ determined in sugars and pulp, the fact that values above the maximum limit proposed by AIJN were found means that there is the risk that these certainly genuine samples could be misjudged as adulterated by the addition of sugars derived from C4-plants. On the contrary, the opposite can also happen, and small amounts of sugar can not move $\delta^{13}C_{sugars}$ to values that are outside the AIJN range of variability (Rossmann et al., 1997). Nevertheless, it is worth noting that the parameter most frequently used to detect adulteration with C4 plant-derived sugars is $\delta^{13}C_{ethanol}$, especially combined with $(D/H)_I$, whose AIJN maximum threshold value was observed in this study.

Tomato passata

Tomato passata with Brix between 7.5 and 11.9 showed $\delta^{18}O_{water}$ values in the range -1.4 and 4.8‰ . To define the minimal threshold value acceptable for an authentic passata, the 95% reference interval of passata $\delta^{18}O_{water}$ values was calculated as mean ± 2 *standard deviation, with 95% of the data falling in the range -1.0‰ to $+3.6\text{‰}$. However, in order to be more conservative, as two certainly genuine passata showed values lower than -1.0‰ , the real minimum value determined in the dataset (-1.4‰) was taken into account as the minimum threshold value. On the other hand, passata samples not subjected to evaporation presented $\delta^{18}O_{water}$ values in the range -4.5‰ and $+2.8\text{‰}$, with eleven out of 120 samples having values lower than the proposed value of -1.4‰ . So, the previously suggested lower threshold value is not entirely applicable to this class of products, but this should be considered bearing in mind that tomato passata commercially available generally has Brix values of about 7.5-8, for reasons of consumer taste, whereas non-evaporated products normally have lower Brix values. As the Ministerial Decree can be interpreted in two different ways, it is necessary to clarify whether it is possible to differentiate passata samples that have previously been concentrated up to 11.9 Brix, from products obtained with watered down paste

(original Brix higher than 12). In this study, diluted samples of passata were highly statistically different from the four diluted paste classes and in particular the passata data did not overlap at all with the distribution of paste, double and triple concentrated paste groups. 95% of the values (mean \pm 2*standard deviation) for diluted samples of passata fell in the range -2.9‰ to -0.4‰, and considering -2.9‰ as the lower threshold limit, all diluted passata samples were over this limit (range of values -2.8‰ to 0.9‰). For this reason -2.9‰ could be proposed as a suitable lower reference value for passata obtained from the dilution of tomato products with Brix value of up to 11.9. It is important to note that although diluted passata was not completely differentiable from diluted semi-concentrated tomato paste, it was completely distinguishable from concentrated paste, and largely from double and triple concentrated paste. Indeed, the counterfeiting of passata occurs using these last two types of products, as they are more economic to import.

The two proposed lower threshold values are particularly effective as they have been obtained from a fairly extensive database that includes the main Italian production areas and different harvests, therefore referring to different climatic conditions, which are one of the most important variability factors for the $\delta^{18}\text{O}$ of vegetal water.

Conclusions and final outcomes

Citrus juices

The comparisons done in this study showed that the AIJN thresholds are some times not fully applicable to samples of Italian origin. However, it is worth noting that these limits are considered to be 'relevant for the evaluation of the identity and authenticity' of a product but must be 'subject to expert interpretation'.

In light of the obtained results, Dr. Federica Camin contacted AIJN since March 2014 asking a revision of the threshold limits. Upon AIJN request, data of authentic samples collected by the inspectors of Italian Ministry of Agriculture in the seasons 2010 - 2014 were sent to AIJN for consideration (Email 16/12/2014). After several reminders Dr. David Hammond (VP of AIJN expert Group) answered that the experts group agreed in adding a commentary note updated on the Italian data sent by FEM. However, up to now, the commentary note has not been added to the AIJN Code of Practice (last remind sent 19/10/2016).

Tomato juice and passata

Similarly to citrus juices, the comparisons done in this study showed that the AIJN thresholds are sometimes not fully applicable to samples of Italian origin also in the case of tomato juices. Therefore, in the email of 16/12/2014 also the data collected from 2010 to 2013 for authentic tomato juices (about 340 samples officially collected by the inspectors of Italian Ministry of Agriculture) were sent to AIJN. In the same file of tomato juice, also a sheet with data of around 150 of authentic samples of tomato sauce (tomato 'passata', Brix degree from 7.5 to 11.9) officially collected were added. In this last case we asked to introduce this kind of product in the AIJN guidelines. As for citrus juice, up to now, the commentary note has not been added to the AIJN Code of Practice (last remind sent 19/10/2016).

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Geographic origin of wheat defined by Sr isotopes ratio and elemental analysis

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Background

Food traceability is an increasing topic for public and private institutions; market globalization introduced great variety of commodities and frequently the consumers want to be sure about the geographical origin of the product.

The detection of geographical frauds could preserve the quality, but also the safety of the food.

An innovative analytical tool widely used to assess food origin is the heavy isotopes ratio evaluation: the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio is one of the most robust and precise parameters for the geographical discrimination because it is only influenced by the age and the pedological characteristics of soils, and it is not influenced by atmospheric events or by man interventions (while it happens using light isotopes like C, O or H).

Thanks to this parameter, it is possible to assign a precise isotopic fingerprint to an agricultural area, or to distinguish through different geographical origins.

From an analytical point of view, a critical issue is the separation between ^{87}Rb and ^{87}Sr : these two elements have approximately the same accurate mass and a discrimination is not possible. For this reason, sample pretreatments with ion exchange resins or ion chromatographic techniques are required in order to avoid signal overlay.

Case Study: geographical discrimination of wheat with $^{87}\text{Sr}/^{86}\text{Sr}$ evaluation

The aim of this work is the development of an analytical method able to discriminate the geographical origin of wheat by using Sr isotopes and elemental analysis.

Strontium isotopes will be detected and quantified with a Multicollector ICP-MS and subsequently a chemometric study will be performed.

Novelty statement

The novelty of the study is the application of a heavy and unconventional stable isotopes analysis coupled with an elemental analysis to an industrial scale in put under control the origin of wheat in the supply chain.

Experimental

Fifty wheat samples coming from different Italian regions, USA, Canada, Russia, Greece and Turkey have been analyzed.

The Sr isotope analysis was performed by a MC-ICP-MS. We also did an elemental analysis, to determine the concentration of 20 elements that help to discriminate the origin of the samples.

Results & Discussion

The method is able to clearly discriminate wheat by national origin: all the samples from a specific country are well separated from the others, in particular if combined with elemental analysis. The separation among Italian regions is more difficult using only Sr isotope but in any case, sample coming from the south of Italy can be separated from the others.

- Mexico

We have only two samples in Mexico, but they show very coherent data. Total Sr and $^{87}\text{Sr}/^{86}\text{Sr}$ work in order to separate them from all the other data.

- Greece and Turkey

Greece and Turkey should be separated from the other data based on Mn and $^{87}\text{Sr}/^{86}\text{Sr}$.

We have three samples of Greece North, and they show a $^{87}\text{Sr}/^{86}\text{Sr}$ around 0.7095 and Mn concentration between 25 and 30 mg/kg. The Greece South sample is different from those coming from North, showing a Sr isotope ratio around 0.709.

The four Turkey samples show a Sr isotope ratio between 0.7075 and 0.7085; the two samples coming from Anatolia and the two coming from Central Turkey show very similar values. At the same time, in the Anatolian samples the Mn concentration is between 25 and 30 mg/kg, while in the samples coming from Central Turkey the Mn concentration is between 35 and 40 mg/kg.

- South Italy

We have twelve samples coming from south Italy. Nine of them are included in the range 0.7079 and 0.7085 for the Sr isotope ratio, while their Sr concentration is between 1 and 4 mg/kg.

The samples from the South are well distinguished from all the other samples; we have three samples that significantly differ from this pattern showing. The nature of these samples is under scrutiny and the soil quality of the areas where they have been taken is actually under study.

- Central and North Italy

We have six samples in the North and eighteen in the central Italy. It's difficult to discriminate, at the moment, which samples are from which area because of the overlap of the data.

Sr isotopes ratio and Sr concentration appear to be linear correlate in the Central Italy samples.

The North Italy data seems divided in two distinct clusters spread around Central Italy data, probably representing soils with different geological origin; this hypothesis is actually under study.

Final Outcomes

Using this method, we are able to distinguish the geographical origin of almost all samples, in particular combining the Sr isotopic fingerprint and the elemental analysis. The parameters we used and the results are different for each different analyzed area. The results gathered at the moment are reported in the following table where the green highlighted cells represent the element that distinguish that geographic origin from the others. The study is in progress to increase robustness of the model and to define another isotopic element to increase the differentiation of geographical origin.

Country/Region	Chemical		
	Sr isotope ratio	Sr	Mn
Mexico			
Greece			
Turkey			
North Italy			
Central Italy			
South Italy			

Tab. 1: results obtained

