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Towards a harmonized approach for food authenticity marker validation and accreditation

Stéphane Bayen*, McGill University, Department of Food Science and Agricultural Chemistry, 21111 Lakeshore, Ste Anne de Bellevue, Quebec, Canada, stephane.bayen@mcgill.ca +1-514-398-8618, ORCID 0000-0002-9935-6685

Chris Elliott*, Queens University Belfast, Queen’s University Belfast, Institute for Global Food Security, School of Biological Sciences, 19 Chlorine Gardens, Belfast, UK; School of Food Science and Technology, Faculty of Science and Technology, Thammasat University, 99 Mhu 18, Pahonyothin Road, Khong Luang, Pathum Thani 12120, Thailand, Chris.Elliott@qub.ac.uk +44(0)2890974721, ORCID 0000-0003-0495-2909

Marco Arlorio, University of Piemonte Orientale “Amedeo Avogadro”, Department of Pharmaceutical Sciences - Food Chemistry, Biotechnology and Nutrition Unit, Largo Donegani 2, Novara (IT), marco.arlorio@uniupo.it, +39 0321-375772, ORCID 0000-0001-5114-7203

Nicolai Zederkopff Ballin, Danish Veterinary and Food Administration, Department of Food Chemistry, Soendervang 4, 4100 Ringsted, Denmark, nixb@fvst.dk, +45 7227 6162, ORCID 0000-0002-9179-9975

Nicholas Birse, Queen’s University Belfast, Institute for Global Food Security, School of Biological Sciences, 19 Chlorine Gardens, Belfast, UK, n.birse@qub.ac.uk, +44 2890 975564, ORCID 0000-0002-3867-3273
Jens Brockmeyer, University of Stuttgart, Institute of Biochemistry and Technical Biochemistry, Department of Food Chemistry, Allmandring 5b, Stuttgart, Germany, jens.brockmeyer@lc.uni-stuttgart.de, +49 711 685 64359, ORCID 0000-0002-8671-5204

Shawninder Chahal, McGill University, Department of Food Science and Agricultural Chemistry, 21111 Lakeshore, Ste Anne de Bellevue, Quebec, Canada, shawninder.chahal@mail.mcgill.ca, ORCID 0000-0003-0627-8006

Maria G. Corradini, University of Guelph, Department of Food Science and Arrell Food Institute, 50 Stone Rd E, Guelph, ON, N1G 2W, Canada, mcorradi@uoguelph.ca, +1-519-824-4120 x 5-3344, ORCID 0000-0001-6844-6437

Robert Hanner, University of Guelph, Department of Integrative Biology and Arrell Food Institute, 50 Stone Rd E, Guelph, ON, N1G 2W, Canada, rhanner@uoguelph.ca, +1-519-824-4120 x53479, ORCID 0000-0003-0703-1646

Stephan Hann a, b, a: University of Natural Resources and Life Sciences (BOKU), Department of Chemistry, Institute of Analytical Chemistry, Muthgasse 18, 1190, Vienna, Austria, ; b: FFoQSI GmbH, Technopark 1D, 3430 Tulln an der Donau, Austria, stephan.hann@boku.ac.at, +43 1 47654 77191, ORCID: 0000-0001-5045-7293

Kristian Holst Laursen, University of Copenhagen, Department of Plant and Environmental Sciences, Thorvaldsensvej 40, 1871 Frederiksberg C, Copenhagen, Denmark, holst@plen.ku.dk, +453533728, ORCID 0000-0001-7900-3324

Alina Mihailova, Food Safety and Control Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Department of Nuclear Sciences and Applications,
International Atomic Energy Agency, Vienna International Centre, PO Box 100, 1400 Vienna, Austria, a.mihailova@iaea.org, +43-1-2600-28373, ORCID 0000-0003-3820-9830

**Teresa Steininger-Mairinger** a,b, a: University of Natural Resources and Life Sciences (BOKU), Department of Chemistry, Institute of Analytical Chemistry, Muthgasse 18, 1190, Vienna, Austria; b: FFoQSI GmbH, Technopark 1D, 3430 Tulln an der Donau, Austria, teresa.mairinger@boku.ac.at +43 1 47654 77186, ORCID: 0000-0001-7809-1529

**Michele Suman**, a,b. a Barilla G. e R. Fratelli S.p.A., Analytical Food Science, Via Mantova, 166, 43122, Parma, Italy. b Catholic University Sacred Heart, Department for Sustainable Food Process, Piacenza, Italy, michele.suman@barilla.com, +39 0521 262332, ORCID 0000-0003-0719-6627

**Lei Tian**, McGill University, Department of Food Science and Agricultural Chemistry, 21111 Lakeshore, Ste Anne de Bellevue, Quebec, Canada, lei.tian@mail.mcgill.ca, ORCID 0000-0002-0929-6501

**Saskia van Ruth**, University College Dublin, School of Agriculture and Food Science, Belfield Campus, Dublin 4, Ireland, saskia.vanruth@ucd.ie, +353 1 716 2832, ORCID 0000-0003-3955-7976

**Jianguo Xia**, McGill University, Institute of Parasitology, 21111 Lakeshore, Ste Anne de Bellevue, Quebec, Canada, jeff.xia@mcgill.ca, ORCID 0000-0003-2040-2624

* corresponding authors
Abstract

Recent publications in the field of food authentication have reported using analytical methods which measure changes in sample composition. These changes can be due to a variety of causes such as the presence of adulterants, different production methods, or varying geographical origins of food. While the increasing use of marker-based approaches is beneficial in combating food fraud, there is a pressing need to adopt a harmonized approach for validating these markers. In this article, we make recommendations for harmonized terminologies and general definitions related to food authenticity markers. First, we propose the terms “primary” and “secondary” markers to distinguish between direct and indirect authentication. The terms “single” and “dual” authenticity markers, and authentic “profiles” and “fingerprints” are suggested to distinguish between the number of analytical targets used. We also recommend that the terms: “threshold”, “binary”, and “interval” markers are applied depending on how they discriminate authentic from non-authentic samples. Second, we advocate for harmonization in marker discovery approaches. A summary of the main analytical techniques, published guidelines, data repositories and data analysis approaches is presented for various marker classes while also stating their applicability and limitations. Finally, we propose guidelines for the analytical community concerning marker validation. In our view, the validation of the authentication method should include the following steps: 1) applicability statement; 2) experimental design; 3) marker selection and analysis; 4) analytical method validation; 5) method release; 6) method monitoring. Implementing these approaches will represent a significant step towards establishing a wide range of fully validated and accredited methodologies that can be applied effectively in food authenticity monitoring and control programs.
Keywords

accreditation; classification algorithm; food fraud; foodomics; machine learning; non-targeted analysis
1. Introduction

Concerns regarding the authenticity of various food types have existed for thousands of years, leading to efforts to detect different types of counterfeiting including adulteration and tampering in food products throughout history (Kowalska et al., 2018; Spink et al., 2013). Analytical chemistry has played a crucial role in forensic investigations related to food fraud, with significant milestones occurring over the past few centuries. Two pivotal events were the introduction of the 1860 Act in the United Kingdom, known as the Prevention of the Adulteration of Food or Drink, which led to the establishment of Public Analyst Laboratories, and the 1906 Pure Food and Drugs Act in the United States, which laid the foundation for what would become the Food and Drug Administration. These initiatives were prompted by widespread public health issues caused by various fraudulent practices in the food industry. Food fraud can negatively impact all stages of the supply chain, from producers and manufacturers who struggle to compete with offenders to consumers who are paying for substandard products. This can undermine consumer confidence, increase public health risk, lead to economic destabilization and have legal consequences.

Adulterated or mislabeled products can contain toxic substances, food allergens, or other harmful contaminants that may result in foodborne illness, allergic reactions or, in some cases, even death. Different analytical methods were developed and applied to detect known adulterants, such as alum in bread or lead in cheese, depending on the available equipment. These tests were developed, validated, accredited, and implemented in food authenticity control programs in many countries.

Recent public outrages, such as the 2008 China melamine crisis and the 2013 European Horsemeat scandal, have further highlighted the need for improved food authenticity detection methods (Gossner et al., 2009; Kowalska et al., 2018). The China melamine incident, in particular, was particularly intriguing from an analytical chemistry standpoint, as it involved deceiving a standard
milk quality testing method by adulteration with melamine (Gossner et al., 2009). This led to a realization in the scientific community that alternative measurement approaches were necessary to detect a wide range of frauds that could evade conventional analytical methods. As a response, various "systems biology approaches" were explored with varying degrees of success.

For instance, metabolomics, a concept introduced in the late 20th century (Oliver et al., 1998), has since found applications in various life science research areas, and more recently, food authenticity analysis. The term "foodomics" was coined to describe one of the applications (Herrero et al., 2012), and numerous publications have since employed foodomics-type approaches such as non-targeted lipidomics and metabolomics. These approaches involve indirect measurements of markers indicating adulteration or a holistic pattern recognition of changes in sample composition due to adulterants, different production methods, or varying geographical origins of food.

While the increasing use of marker-based approaches is beneficial in combating food fraud, there is a pressing need to adopt a systematic and harmonized approach for validating these markers. Without such an initiative, accrediting these methodologies will be extremely challenging, hindering their routine use in monitoring and control programs. Markers can stem from various systems biology approaches, including elements, metabolites, lipids, proteins, and nucleic acids. However, each approach poses reliability challenges, as demonstrated by the limited clinical use of identified cancer biomarkers due to validation issues (Diamandis, 2012). We must avoid a similar outcome in food authenticity marker approaches.

Therefore, achieving a consensus and harmonized approach for marker validation and accreditation is crucial. Valuable lessons can be learned from other systems biology approaches in different research domains, such as proteomics (Calderón-Celis et al., 2018; Deutsch et al., 2017) and metabolomics (Emwas et al., 2016; Sumner et al., 2007; Xia et al., 2013).
In this review, we focus on three critical areas where harmonization is essential for food authenticity marker approaches. First, we emphasize the need for standardized terminologies and definitions in the field. Second, we advocate for harmonization in marker discovery approaches. Finally, we propose guidelines for the analytical community concerning marker validation. Implementing these approaches will represent a significant step towards establishing a wide range of fully validated and accredited methodologies that can be applied effectively in forensic food authenticity monitoring and control programs, providing accurate and reliable data to support enforcement actions.

2. Harmonization of terminology and definitions

Food authentication involves expertise from multiple scientific fields, such as analytical chemistry, food and agricultural science, molecular biology, statistics, data science, and many others. This inherent multi-disciplinary practice has contributed to a lack of consistency in the terminologies being used in the scientific literature to describe authenticity markers and analytical workflows for food authenticity testing.

Numerous keywords have been used by researchers to describe food authenticity markers such as: “analytical marker”, “biomarker”, “discriminant marker”, “analytical target”, “signature”, “profile”, “pattern”, “fingerprint”, “blueprint”, etc. Depending on the analytical technique being used, markers can be classified as chemical, molecular, biological, biochemical, etc. Furthermore, authenticity testing can be performed using targeted, non-targeted, or hybrid approaches which further complicates nomenclatures selected to describe authenticity markers.

The principles, nomenclature, and applications of targeted and non-targeted analytical approaches for food authentication have been discussed in several comprehensive reviews (Ballin & Laursen,
2019; McGrath et al., 2018; Medina et al., 2019; Mialon et al., 2023; Popping et al., 2022). The terms “non-targeted”, “nontargeted”, “un-targeted”, and “untargeted” have been used interchangeably. In addition, the terms “profile”, “signature”, “pattern”, and “fingerprint” have been used inconsistently by different authors. For example, some studies use the term “non-targeted profiling” instead of fingerprinting (Chen et al., 2017; Cubero-Leon et al., 2014), while others refer to “targeted fingerprinting” as opposed to profiling (Magagna et al., 2016; Stilo et al., 2019). Other groups decided not to distinguish between profiles and fingerprints (Sánchez-Salcedo et al., 2016). Furthermore, some studies have used “elemental profiles” (Li et al., 2013; Zhao & Zhao, 2019) and “elemental fingerprints” (Chen et al., 2009; Laursen et al., 2014; Laursen et al., 2011) synonymously.

There is an urgent need for the field of food authenticity analysis to adopt harmonized terminologies, standardized validation protocols and analytical workflows from sample preparation through to data processing, interpretation, and reporting. These regimes are particularly lacking in non-targeted analyses. The need for harmonization and standardization has been emphasized by multiple research groups (Ballin & Laursen, 2019; McGrath et al., 2018; Nichani et al., 2023), and further attempts towards harmonization have been made by the USP (2018) and the AOAC (2020) as well as by international collaborative projects (e.g. the Food Integrity Project; European Union’s 7th Framework Programme, KBBE.2013.2.4-01).

Here we make recommendations for harmonized terminologies and general definitions related to food authenticity markers. We have adopted the terms “primary” and “secondary” markers, as proposed by Ballin & Laursen (2019). Figure 1 graphically presents examples of the most widely used terms and provides the corresponding recommended harmonized terminologies for authenticity markers in targeted and non-targeted authenticity testing.
An authenticity marker is a distinctive indicator that can be used to directly or indirectly verify product characteristics that correspond to claims made about the product. An authenticity marker must be capable, individually or in combination with other markers, of discriminating between an authentic and a non-authentic product.

A primary authenticity marker is a distinctive indicator that can directly verify product characteristics that correspond with a product claim (direct authentication). Primary markers are evaluated using targeted analysis and often rely on legal limits, though the absence of legal limits for many markers that would be of interest can reduce the usefulness of this approach at times. Primary markers are widely used in the case of food adulteration, e.g., determination of melamine in milk powder (Filazi et al., 2012).

A secondary authenticity marker is a distinctive indicator that can indirectly verify product characteristics that correspond with a product claim. Secondary markers are evaluated using targeted analysis and are often evaluated using internationally acknowledged threshold values or conversion factors. However, the analysis of secondary markers can only offer an estimate for authentication (indirect authentication). An example of secondary markers can be the application of stable isotope ratios, multi-element or fatty acid profiles for the verification of authenticity and geographical origin of olive oil (Alves et al., 2016).

A single authenticity marker is a marker that uses a single analytical target to discriminate between authentic and non-authentic samples.

A dual authenticity marker is a marker that uses two analytical targets to discriminate between authentic and non-authentic samples. An example of a dual authenticity marker can be enantiomer ratios or the sum concentrations of two compounds (Xu et al., 2022).
An authentic profile is a result of the measurement of multiple specified analytical targets. The exact number of analytical targets, which constitute a profile, is not internationally harmonized. Here we adopt the recommendation of Ballin & Laursen (2019), who suggested that more than two targets are required to use the term “profile”. The terms “signature” and “pattern” are suggested to be synonyms for “profile”.

An authentic fingerprint is the result of a non-targeted analysis (NTA) which offers simultaneous detection of numerous unspecified data points, often more than one hundred. Fingerprints can be composed of nucleic acid fragments, multidimensional comprehensive chromatographic profiles, spectral or mass spectrometric peaks, or NMR/UV-Vis/luminescence bins whose underlying IDs are not yet known. This analysis is particularly important when no primary or secondary markers are available.

In the context of the proposed definitions, the term “analytical target” is suggested to be synonymous with “measurand”. On the other hand, the term “feature” would be applicable in the case of certain fingerprints, for example, the analysis of morphological features in multispectral imaging. However, for simplicity and harmonization we suggest that “analytical target” should be used.
Figure 1: Examples of the most widely used terms in analytical authenticity testing (left) and the recommended harmonized terminologies for authenticity markers in targeted and non-targeted analysis (right). A red bullseye represents a primary marker, blue bullseye represents a secondary marker, and the fingerprint illustrates that no specified markers are targeted but that the measured analytical targets/data points are still within a closed entity, e.g., proteomics, metabolomics, etc. (modified from Ballin & Laursen (2019)). Made with BioRender.
In addition to harmonized terminologies, it is essential to have a harmonized classification of authenticity markers that can be adopted for the development, validation, and technology transfer of analytical methods for food authentication. In the following section we propose such classification of markers based on the manner by which they discriminate authentic from non-authentic samples and show examples of their use cases for different authentication issues. In particular, we recommend that the terms: threshold, binary, and interval marker are applied going forward. In addition, we differentiate between the terms linear and non-linear profile or fingerprint (Figure 2).

A **threshold marker** is an authenticity marker whereby a threshold exists that can discriminate between an authentic and non-authentic product. For example, differences between $^{13}\text{C}/^{12}\text{C}$ isotope ratios of protein and sugar compounds in honey greater than 1‰ would reflect adulteration with invert sugar syrups (Elflein & Raezke, 2008).

A **binary marker** is an authenticity marker whereby its presence or absence can discriminate between an authentic and non-authentic product. A binary marker is an extension of a threshold marker, whereby the threshold is set at the limit of detection (LOD). For example, the detection of a prohibited synthetic adulterant, or the detection of DNA markers for *L. scoparium* in New Zealand manuka honey (McDonald et al., 2018).

An **interval marker** is an authenticity marker whereby an interval exists such that authentic samples fall within the interval and non-authentic samples are outside, or *vice versa*. For example, for polyfloral manuka honey, 3-phenyllactic acid should be in the 20-400 mg/kg range (McDonald et al., 2018).
Fingerprints are evaluated using multivariate statistics or machine learning algorithms from non-targeted approaches. They can be further classified as linear or non-linear based on the algorithms used.

A linear profile or linear fingerprint is an authentic profile or fingerprint, respectively, whereby authentic samples are distinguished from non-authentic samples by linear methods. Examples of linear machine learning classifiers include logistic regression, support vector machine (with linear kernel), and partial least squares discriminant analysis (PLS-DA). In essence, a linear profile or fingerprint is a threshold marker applied to the weighted sum of all the profile or fingerprint's features.

A non-linear profile or non-linear fingerprint is an authentic profile or fingerprint, respectively, whereby authentic samples are distinguished from non-authentic samples by the application of non-linear statistical methods. Examples of non-linear machine learning classifiers include artificial neural networks, support vector machines (with radial basis function kernel), k-nearest neighbours, and random forest.

Recognizing the diversity of markers is essential for marker discovery, as further discussed in the next section. For example, mining large data sets for threshold, binary, or interval markers will require different algorithms. Finally, it is worth mentioning here that the different types of markers can be used together to create a profile that increases the difficulty and cost of committing fraud. A case study of this being the authentic profile used to authenticate manuka honey (McDonald et al., 2018).
Figure 2: A comparison of the various marker types. An example is shown for a single authenticity marker being used as a: a) threshold, b) binary, and c) interval marker. Similarly, an example is shown of an authentic profile of three analytical targets being used as a d) linear profile and e) non-linear profile. The non-linear profile can take on many forms beyond that shown here.
3. Harmonizing marker discovery

There are a multitude of compound classes to test for their suitability as authenticity markers. Deciding on the appropriate compound class(es) to investigate depends on the context of how the respective food product could be authenticated. For example, examining isotope ratios allows verification of the geographical origin (Mazarakioti et al., 2022), and studying profiles of small molecules, e.g., lipids or sugars, helps to derive changes in composition (Kalogiouri et al., 2020) or detect adulterants (De Angelis et al., 2021). Evaluating each analytical target type’s capacity to act as a marker can also render a combination of different marker classes necessary (Walker et al., 2022). In this context, data fusion, i.e., the integration of multiple complementary data sources to produce more consistent, accurate, and useful information than from an individual data source (Klein, 2004), has proven to be a powerful tool for authentication (Jandric et al., 2021).

The selection of a suitable marker compound(s) is based on analytical scouting techniques and data post-processing by appropriate statistical methods and in some cases data fusion. Non-targeted analysis is particularly suited for marker discovery since the identity of the marker does not need to be known prior to starting the analysis and aims at the potential to find profiles rather than an individual marker (Dunn et al., 2013). Candidate markers can be extracted from these patterns and evaluated through targeted methods. The selected compounds (individual, combinations, or whole profiles) are used in either a qualitative or a quantitative manner and thereby allow for unambiguous verification of a product’s authenticity (e.g., Creydt et al. (2022)).

Analytical scouting techniques and data handling – how to analyze specific marker classes

Elements and isotopes: Elemental/isotopic analysis is nowadays mainly performed with mass spectrometric techniques using a range of ionization sources. For the measurement of stable
isotope abundance ratios of low atomic mass elements (see Table 1), isotope ratio mass spectrometry (IRMS) is often the method of choice (Monti et al., 2023). In contrast, isotopes of high atomic mass elements are analyzed by thermal-ionization mass spectrometry (TIMS) or multicollector inductively coupled plasma mass spectrometry (MC ICP-MS). ICP-MS with sector field or quadrupole mass separation and single detectors is utilized when it comes to quantitative or relative quantitative approaches aiming at the measurement of multi-element concentrations or elemental patterns, respectively.

The main advantage of isotope ratio-based marker analysis is the high precision achievable under repeatability conditions of measurement (< 0.05% RSD) and the possibility to correct bias with matrix-matched certified reference materials (CRMs), leading to highly accurate results. Moreover, the assessment of authenticity has already been regulated for several commodities by metrological authorities and is available as ISO or AOAC standards, which can be implemented into the scope of accredited food testing laboratories (e.g., AOAC 984.23, OIV-AS312-07, EN 17958, OENORM ENV 12140, OENORM ENV 12141, OENORM ENV 12142).

**Small molecules:** Analytical techniques aimed at the measurement of small molecules, i.e., compounds with a molecular weight below 1500 Da, are challenged by the physico-chemical diversity of this marker class (Mihailova et al., 2021). Polarity is typically the critical parameter; here, lipidomics emerged from metabolomics as an independent research area (Sun et al., 2020). Even if the analytical scope is differentiated between polar and non-polar compounds, a single analytical method is not able to cover the respective range of compounds. Hence, when decisions on sample preparation, separation, and compound detection techniques are made, the inherent strengths and limitations of each methodology need to be considered carefully.
Within the last decade, employing chromatographic separation, including multidimensional chromatography for enhanced separation performance and profiling, coupled with high-resolution mass spectrometry for a NTA approach has proven an excellent scouting technique (Cuadros-Rodríguez et al., 2021). Starting with a myriad of signals, data is pre-processed to group individual spectral m/z peaks to an individual compound signal, including a molecular formula-dependent isotopic pattern. After statistical analysis, the data set is funneled to a manageable number of small molecules to be identified (in terms of their chemical structure) or combined with other compounds to be further investigated for their suitability as a profile. An NTA approach is a powerful scouting tool. However, it is inherently challenging to validate since analytical performance characteristics, i.e., trueness, precision, and robustness against different matrices or varying environmental conditions, can only be straightforwardly assessed for identified compounds. Apart from MS-based techniques, spectroscopic methods, e.g., Raman, UV-Vis, fluorescence or NMR, IR spectroscopy (NIRS, MIRS), and sensor technologies (e-Nose, e-Tongue, etc.) are applied for the analysis of small molecules (Lohumi et al., 2015; Mialon et al., 2023; Peris & Escuder-Gilabert, 2016; Sobolev et al., 2017).

**Peptides and proteins:** Proteins (and their peptides) constitute valuable markers of food authenticity, providing information on varieties/species, adulteration and processes that a food might undergo, such as enzymatic modifications, heat treatments, or freezing and thawing cycles (Sun et al., 2020). Gel-based electrophoresis techniques have traditionally been used to characterize and identify protein profiles for food authentication based primarily on their electrophoresis patterns (e.g., using isoelectric focusing). However, in recent years, MS-based methods have become the tool of choice for identifying specific peptide and protein markers that can contribute to food authentication due to their significantly higher throughput, sensitivity, and
specificity. Currently, bottom-up and top-down workflows are applied to systematically explore
and identify novel marker proteins/peptides and to collect fingerprints. Bottom-up approaches
employ enzymatic digestion of the proteins to peptides, in general using trypsin. This step further
increases the number of analytical targets in non-targeted approaches, but bottom-up approaches
benefit from excellent chromatographic separation of peptides and efficient peptide fragmentation
in MS/MS mode. Due to the proteome complexity in food samples, pre-fractionation at the protein
or peptide level might be applied followed by separation and analysis. Notably, bottom-up
approaches allow multiplex analyses such as multiplex species identification in complex foods, or
the combination of suitable analytical tasks (e.g., species identification and allergen analysis)
(Korte & Brockmeyer, 2017). Top-down workflows in food proteomics and food authenticity
testing rely mainly on Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass
Spectrometry (MALDI-ToF MS) protein fingerprinting approaches and deliver fast non-targeted
approaches, e.g., for species identification. However, one drawback is processing-induced protein
modification and mass shifts due to exposure to high temperature and high vacuum. Despite the
advantages of proteomics in the discovery of potential markers for food products and ingredient
authenticity, this class of markers is the least explored to date. First approaches were made to
transfer methods to routine analysis (Jira et al., 2022), and further advances in instrumentation and
bioinformatics tools to assist data interpretation are likely to play a significant role in increasing
the discovery of peptide and protein markers for food authentication.

**Nucleic acids (DNA/RNA):** Nucleic acid (DNA/RNA) sequences are routinely used for food
authenticity, traceability, and safety testing. DNA sequences can be considered almost impervious
to extrinsic factors such as weather and agricultural practices (Creydt & Fischer, 2020) while RNA
sequences can provide important information on viability (e.g., of pathogens). Several
methodologic approaches involve polymerase chain reaction (PCR) and/or sequencing technologies, all of which rely on reference (e.g., sequence) databases for their application. The latter require careful curation, harmonization, and version control to be of broad utility in food forensics and while these issues are beyond the scope of the current manuscript, they must be addressed for DNA-based methods to be deemed fit-for-purpose in food authentication studies. Genetic mutations (insertions, deletions, or substitutions) drive the diversification of genomes over time and because different genes have different rates of molecular evolution, comparative analysis of aligned (e.g., homologous) nucleotide sequences are used to identify genomic regions (e.g., genetic markers) suitable for identification of one or more organismal lineages (populations, species, higher taxa). For example, sequence motifs that are conserved across many phyla can be used to develop “universal PCR primers” that can identify a broad range of species, while sequence motifs that are unique to a given species can be used to develop “species specific” PCR primers. The latter are typically employed using various PCR technologies (e.g., endpoint PCR, quantitative PCR, digital PCR) to screen for a particular species of interest while the former are used to identify one or more species using various sequencing technologies (e.g., Sanger “DNA Barcode” sequencing of discrete tissues, high-throughput “metabarcode” sequencing of complex mixtures) by matching sequences with entries in the reference sequence library. Here we refer to these methods as “targeted” because they employ PCR primers to amplify a specific genetic marker gene region. In the case of multiplexed qPCR assays and metabarcoding, we refer to these methods as “multi-targeted”. We refer to “non-targeted” methods as those that rely on random PCR amplification of genetic variants (e.g., RADseq) or PCR-free high-throughput sequencing (e.g., shotgun sequencing). Targeted approaches are most commonly used in food authentication at this time because reference sequence libraries are most densely populated for specific marker gene
regions (e.g., mitochondrial cytochrome c oxidase I “DNA Barcodes” and other markers noted by Ballin et al. (2019)) and because they include multiple individuals of each species to support an assessment of intra vs interspecies variation, which is necessary to assess the specificity of molecular methods.

Table 1 summarises the main analytical techniques, published guidelines, data repositories and data analysis approaches for each marker class while also presenting their applicability and limitations. It should be noted that Table 1 is not meant to be exhaustive. Instead, it provides a starting point for each marker class, emphasizing the most relevant techniques, preparation methods and examples of guidelines and reference repositories.
Table 1. Potential marker classes, analytical techniques, requirements and uses.

<table>
<thead>
<tr>
<th>MARKER CLASS</th>
<th>ANALYTICAL TARGET</th>
<th>ANALYTICAL TECHNIQUE</th>
<th>DATA PROCESSING</th>
<th>DATA ANALYSIS &amp; MODELING</th>
<th>APPLICATIONS</th>
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| ELEMENTS/ISOTOPES | **Common Isotopes:** $^3$H, $^1$H; $^{13}$C,$^{12}$C; $^{15}$N,$^{14}$N; $^{18}$O,$^{17}$O,$^{16}$O; $^{36}$S, $^{34}$S, $^{33}$S, and $^{32}$S | **Main Techniques:** IRMS, ICP-MS (SNIF-) NMR | Baseline correction Quality checking Noise filtering Missing value estimation Normalization | **Dimensionality reduction:** PCA, CA, LDA, CDA, PLS-DA, OPLS-DA, SIMCA, etc. | **Uses:**
|               | **Common Ratios:** $^2$H/$^1$H (D/H), $^{13}$C/$^{12}$C, $^{15}$N/$^{14}$N, $^{18}$O/$^{16}$O, $^{34}$S/$^{32}$S, $^{87}$Sr/$^{86}$Sr | **Sample Preparation:** Distillation/Defatting Acid Digestion (Sr) | Standards/Guidelines: EU Regulation 882/2004, Method Validation ISO 5725:1994 | **Classification and Discrimination** Support Vector Machines (SVM), Random Forests (RF), CARTS, ANNs, etc. | **Verification of geographical origin and botanical provenance (beans, seeds)**
|               | **Databases:** Global isotopic for food products (e.g., Global Network of Isotopes in Precipitation (IAEA); Isotope fingerprints compendia | **Repositories (selected examples):** Olive Oil: Italian PDO databank Fruit juice: EU PURE JUICE and TRACE | **Modeling:** Geospatial modeling | **Verification of agricultural practices/factors**
|               | | | | **Detection of fraudulent practices** | **Limitations:**
|               | | | | | **Variations in stable isotopes abundance**
|               | | | | | **Metabolic turnover**
|               | | | | | **Mainly secondary markers**
<table>
<thead>
<tr>
<th>SMALL MOLECULES</th>
<th>Small molecules: sugars, lipids, amino acids, fatty acids, phenolic compounds, alkaloids</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mass range:</strong></td>
<td>50 - 1500 Da</td>
</tr>
<tr>
<td><strong>Databases:</strong></td>
<td>NIST, METLIN, KEGG, PubChem, MassBank, ChemSpider, HMDB, FooDB, MzCloud, Phenol-Explorer, LIPID MAPS, Biological Magnetic Resonance Data Bank</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Main Techniques</strong></th>
<th><strong>Separation:</strong> Chromatography (e.g., GC, LC, UPLC, IC, TLC; 2D-GC, 3D-GC, 2D-LC, 3D-LC)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Detection:</strong> Mass spectrometry (e.g., LC, GC, CE coupled with (HR)MS, MS/MS, TOF, Q-TOF, Orbitrap, FT-ICR; MALDI-TOF, PTR-MS, SIFT-MS, DART-MS)</td>
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<tr>
<td><strong>Vibrational spectroscopy</strong> (e.g., UV/VIS, MIRS, NIRS, Raman, SERS)</td>
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<tr>
<td><strong>Nuclear magnetic resonance spectroscopy</strong> (low and high field NMR)</td>
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<tr>
<td><strong>E-nose, E-tongue, acoustic spectroscopy</strong></td>
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| **Sample Preparation** | (depending on the technique): Homogenization, Freeze-drying, Filtration/Dilute and shoot, Protein precipitation, Phospholipid depletion, Extraction [solid phase (SPE, SPME, DSPE, MSPE), liquid-liquid (LLE), molecular imprinted polymer (MIP)] |

| **Vibrational spectroscopy:** | Exclusion, Filtering, Smoothing, Derivation, Baseline correction, Scatter correction, Normalization |
| **Mass spectrometry-based metabolomics:** | Noise filtering, Baseline correction, Peak picking/peak detection, Alignment, Deconvolution, Normalization, Compound identification |

| **NMR-based metabolomics:** | Zero filling, Phase correction, Baseline correction, Referencing, Alignment, Binning and peak picking, Normalization |

| **Dimensionality reduction:** | PCA, CA, LDA, CDA, PLS-DA, OPLS-DA, SIMCA, etc. |
| **Classification, and Discrimination and Prediction:** | PLSR, OPLSR, SVMs, RF, ANNs, etc. |

| **Uses:** | Verification of geographical origin, Differentiation of species and varieties, Verification of production methods (farmed vs. wild; organic vs conventional), Detection of fraudulent practices (e.g., adulteration of milk with melamine; substitution of Robusta coffee in Arabica) |

<p>| <strong>Limitations:</strong> | Confidence of identification results |</p>
<table>
<thead>
<tr>
<th>Peptide and Proteins: Whole proteome, Subset of proteins, Characteristic Peptides, Structural modification of peptides (e.g. processing-induced modifications)</th>
<th>Main Techniques</th>
<th>Gel-based Electrophoretic Patterns:</th>
<th>Gel-based Electrophoretic Patterns:</th>
<th>Database searching (Servers: Mascot MS-Tag, pepProb, Poptiam)</th>
<th>Statistical Procedures and Bioinformatics Tools</th>
<th>Scoring Calculation</th>
<th>Uses:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel-based: SDS–PAGE, IEF, 2DE electrophoreses; Lab-on-Chip microelectrophoresis MS-based: MALDI-TOF-MS &amp; LC-ESI-HRMS (Peptide Profiling and Mass Fingerprinting) LC-MS/MS (Peptide Fragmentation Fingerprinting, proteomics) LC-MS (SIM or XIC)</td>
<td>Background and noise subtraction Thresholding</td>
<td>Raw data conversion</td>
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<tr>
<td>Preparation Steps:</td>
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<tr>
<td>Uses:</td>
<td>Differentiation of species, varieties, breeds (including GM vs. non-GM) Verification of processing treatments (high &amp; low temperature, high pressure) and storage conditions Detection of fraudulent practices (e.g. transgluatminases)</td>
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| DNA/RNA | DNA/RNA  
Whole Genome, highly conserved target genes | **Main Techniques**  
Sanger Sequencing  
Next-generation Sequencing (NGS)  
PCR-Techniques (e.g., LPA)  
Isothermal amplification techniques (e.g., LAMP, RPA)  
RAPD  
STR  
AFLP-PCR  
DNA barcoding | Sequence Alignment  
Assembled into consensus sequences  
Editing  
Record Removal | Chemometrics methods. |  
**Uses:**  
- Verification of biological identity (including GM vs. non GM)  
- Detection of fraudulent practices |  
**Limitations:** DNA degradation due to extensive processing |
<table>
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<tr>
<th>CRISPR-based nucleic acid assays</th>
<th>High resolution melt analysis</th>
</tr>
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<tbody>
<tr>
<td><strong>Preparation Steps</strong></td>
<td></td>
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<tr>
<td>DNA extraction</td>
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<tr>
<td><strong>Standard/Guidelines</strong></td>
<td></td>
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<td>ISO 21571:2005</td>
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<td><strong>Repositories</strong></td>
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<td>NCBI GenBank</td>
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<td>Barcode of Life</td>
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<tr>
<td>Datasystem</td>
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<tr>
<td>Genetic and Genomic Information</td>
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<tr>
<td>System (GnpIS)</td>
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4. Guidelines for marker validation

Validation of an analytical method should follow procedures such as ISO 17025 (ISO, 2017) or other internationally recognised standards. These standards are sufficient to validate authentication methods with primary markers such as Sudan dyes and melamine where the analytical result can directly be translated to authentic or non-authentic. Often, however, the authenticity questions are more complex and require the use of a combination of secondary markers or a non-targeted fingerprint. In these cases, the analytical results can only indirectly be translated to authentic or non-authentic outcomes, e.g., the case of verification of geographical and/or production origin or adulteration cases where the adulterants are unknown (Bohme et al., 2019; Cardin et al., 2022; de Lima & Barbosa, 2019; Gopi et al., 2019; Katerinopoulou et al., 2020; Lohumi et al., 2015; Medina et al., 2019; Mihailova et al., 2021; Peris & Escuder-Gilabert, 2016; Ye et al., 2023). As a consequence of this indirect translation, additional validation aspects before and after the analytical part are needed to increase the quality and understanding of the final result. We, therefore, propose that validation of an authentication method includes both validation of the marker(s) and validation of the analytical method. This will serve to ease and augment the accreditation process, which is especially challenging in the non-targeted domain. We also believe that this approach will provide the necessary information needed for an accreditation body to accredit the authentication method and not just the analytical part. Figure 3 describes the different steps involved in the validation of an authentication method, with special attention to the marker validation.
Figure 3. The different steps in the validation of targeted and non-targeted authentication methods including applicability statement, experimental design, analysis, method validation, release, and monitoring.
In our view, the validation of the authentication method should include the following steps: 1) applicability statement; 2) experimental design; 3) marker selection and analysis; 4) analytical method validation; 5) method release; 6) method monitoring, all whilst considering the appropriateness of the marker at each stage (e.g., choosing a marker at risk of thermal degradation with an experimental method requiring a high temperature extraction).

Step 1. Applicability statement

In cases of substitution and addition, there will often be a threshold percentage that has been set to discriminate between an unintentional act and a possible intentional act (fraud). This percentage is established by a regulatory authority or a commercial laboratory after considering industrial practices and consumer expectations, such as: i) what levels can be measured analytically with a satisfactory accuracy (trueness and precision)? ii) what is the lowest level of cross-contamination that can be practically achieved in industry? iii) what are the levels and types of cross-contamination encountered and deemed reasonable in terms of good manufacturing practices (GMP)? iv) what is acceptable to a large majority of consumers based on religious, moral, ethical and other grounds? v) what is acceptable from a regulatory perspective in terms of false positive and false negative results? vi) what is the meaningful level in terms of economically motivated adulteration (EMA)?

Step 2. Experimental design

A systematic literature review is a sensible and recommended starting point to gather information about the presence, absence, and range of potential markers across all relevant matrices. If the amount of data available is not substantial, a non-targeted analysis coupled with appropriate data
processing and chemometrics could allow for marker discovery. For targeted approaches, a failure
to know the true identity of the marker(s) can have serious consequences and bias the translation
from the analytical result to the conclusion about authenticity. The potential to identify the
structure of novel biomarkers through use of additional techniques such as NMR, perhaps then
followed by synthesis to enable physicochemical properties to be determined should be considered,
rather than using a biomarker without knowing its chemical structure and formula.
The severity of the identification challenge will be determined by the class of compound selected
as a marker and the presence of different compounds from the same chemical class in non-
authentic samples. Data extracted from the literature must be considered thoroughly and weighted
according to the quality parameters defined in the systematic review. If markers have been
identified, the concentration range in relevant matrices should be determined from the literature
data, if such data is available, otherwise determining the concentration range experimentally is
recommended. Erroneous determination of the concentration of the threshold and interval
marker(s) in different matrices can impede the translation from the analytical result to the
conclusion about authenticity.
Issues in this area require a review of the chosen marker, particularly overlap in concentrations or
inappropriately large uncertainties. When the strategy to go for a targeted or a non-targeted
approach is established, an appropriate number and cross-section of samples that include
adulterated samples and a variety of authentic samples with natural variation must be collected for
later analysis, with effort paid to ensuring representative sampling and demonstrating that the
sampling plans chosen will meet with ISO 17025 or other accreditation approvals where such
accreditation is sought. Natural variation should include geographical location of where the food
has been grown, sampling and processing techniques, season and year of harvest or production,
storage conditions, and the variety/breed of the sample. It is important to define the minimum number of authentic and non-authentic/spiked samples to be used for method development and validation whilst retaining the required levels of statistical significance when undertaking data analysis post data collection. Authentication models should be developed with a sufficiently large and diverse sample size that scales up with the number of features and classes used to develop the model to minimize the risk of overfitting (Vabalas et al., 2019). If a sufficiently large sample size cannot be obtained, then feature selection algorithms, such as recursive feature elimination (Guyon et al., 2002), should be implemented to reduce the number of features. In addition, the study should consider how the samples were prepared and stored and if this reflects the real-world industrial processes that a particular food commodity undergoes.

**Step 3. Marker selection and analysis**

If markers have been identified, an analytical test must demonstrate that the marker identification and concentration agree with the information obtained in the literature review and/or in-house analysis (e.g., in-house marker database), and that the strategy supports the applicability statement. If the analytical measurement indicates that the markers do not support the applicability statement, new markers must be identified. In the absence of identified markers, a non-targeted platform coupled with chemometrics can guide the selection of markers that could subsequently be measured with a targeted platform, with appropriate structural elucidation to support the robustness of the markers being selected.

It should be noted that the cost and validation requirements may decrease when the number of analytical markers selected decreases. However, the number of markers selected must be sufficient to ensure that the method is actually fit-for-purpose in terms of being reliable in discriminating
between authentic and non-authentic samples. A further point worth noting is that having an increased number of markers analysed decreases the chance of fraudsters adding a marker or markers to a type of food to fool the analytical test. Finally, the selection of different numbers and classes of relevant authenticity markers could also be the consequence of collecting more robust statistical information by analysing additional samples over the years. This may be reducing the compounds that are used as markers through iterative refinement. Alternatively, it may be a result of determining better (more reliable) markers as instrument performance increases and the compounds, which are present at very low levels and are capable of giving greater certainty concerning the authenticity, can be detected and quantified reliably.

Step 4. Analytical method validation

The analytical method validation should be performed according to international standards such as ISO 17025 where characteristics include accuracy (bias and precision), limits of detection and quantification, measurement uncertainty, robustness, (e.g., in terms of variation of matrices or laboratory conditions) and selectivity (ISO, 2017). If some of these characteristics do not meet the requirements of the standard, then a new marker selection or the addition of more markers should be pursued. Some of these classical method performance characteristics can be transferred to non-targeted analytical platforms, however, others are not available or applicable (Creydt & Fischer, 2020; Esslinger et al., 2014; Gao et al., 2019; McGrath et al., 2018; USP, 2018). The authors recommend that for the validation of non-targeted methods, performance characteristics include the evaluation of sensitivity and specificity rates obtained by the chemometric model (USP, 2018), which are checked against the applicability statement. A key element in non-targeted methods is the use of databases and the validation of chemometric models. This is of particular importance
when using databases built around theoretical or calculated values, and undertaking some level of validation using reference materials or standards is likely to be both necessary and sensible. Especially transparency is an important element when databases are used to verify the authenticity, and one cannot trust results obtained from hidden information. It is ideal, but not always practical that databases are open for scrutiny. Where possible, non-disclosure agreements can protect the owner of the intellectual rights, though in the case of GMOs or other proprietary food products, it may be the case that chemical compounds found in these products are themselves protected by patents and may not appear within databases.

For both targeted and non-targeted method validation, the assessment of robustness is highly important. If variation is foreseen and necessary for the authentication (e.g., an increase or a decrease in marker concentration that enables one to calculate the age of a food commodity) it should be included in the model.

Step 5. Method release

In fully validated authenticity methods that rely on a primary marker(s) that directly translates to compliance or non-compliance, no further interpretation of the result obtained is needed. On the contrary, if an analytical result requires specialist interpretation before a conclusion about authenticity can be reported, some reporting caveats may be required. Consider the following two examples. 1) Reporting the authenticity of the content of protein derived from the analytical result of nitrogen, could be accompanied with the following text: “The protein content is derived from the measurement of nitrogen with the use of the generally agreed upon correlation factor of 6.25. It is assumed that the vast majority of the measured nitrogen originates from protein. If the product contains added nitorgenous compounds, the result may not reflect the actual content”. 2) Reporting
the authentication of beef from the presence/absence of horsemeat using a DNA analysis could be
accompanied with the following text: “Horse DNA was detected. The DNA can originate from
different animal parts,” or “Horse DNA was not detected. The lack of horse DNA indicates the
absence of horse tissue, but processing can degrade DNA and does not rule out the possibility that
components from horse are present”.

Step 6. Method monitoring

While analytical method validation is generally a one-off process, it is important to ensure that the
authenticity method remains valid over time. This requires regular monitoring of performance
changes and possible drift through the analysis of blind samples, proficiency tests and quality
control samples (preferably CRMs). New samples can also be included to investigate changes in
the food item over the short-term (e.g., seasonal) and long-term (e.g., climate change). For non-
targeted methods, an on-going programme of the analysis of reference samples with additional
metadata might advantageously disclose future relationships, thereby broadening the scope to
include additional authentication traits. Borrowing from the concept of replay memory (Mnih et
al., 2015), the addition of each new sample to the overall sample pool should be followed by the
removal of the oldest sample in the pool. Such an approach ensures that method monitoring is
always performed on a pool of recent samples with an adequate sample size. An alternative
approach is to simply continue adding new samples without removing the oldest sample in the
pool, but while giving greater weight to newer samples and lower weight to older samples. This
can guard against unexpected changes in supply chains, weather patterns, or other aspects of
sample type that aid in determining authentic from non-authentic samples while still placing
greater importance on the newest samples. Moreover, retrospective analysis of the non-targeted datasets may be important in specific cases of food fraud.

5. Conclusion

This article presents several recommendations that we consider crucial for the analytical community involved in developing and validating marker-based approaches to detect food fraud. These recommendations aim not only to identify those responsible for fraudulent activities but also to protect legitimate businesses and consumers from such deceitful practices. It is emphasized that without a well-coordinated and harmonized approach to validate and accredit food markers, progress toward these essential objectives will be significantly hindered.

To address these challenges effectively, a systematic approach is proposed. This approach involves reaching a consensus on the most appropriate terminologies and definitions for the field, harmonizing the methodologies used in marker discovery, and establishing clear guidelines for validating selected markers. Even with the adoption of these best practices, several other obstacles must be overcome to move forward successfully. One significant challenge is the lack of harmonized reference standards and procedures, which complicates the discovery of consistent and universally accepted markers for assessing food authenticity. Furthermore, sharing validated data derived from marker-based authenticity analysis presents difficulties and will require collaboration among research institutions, regulatory bodies, and industry stakeholders. Establishing common standards and practices is essential to create robust databases that can be developed, maintained, and accessed by end users. Considering these challenges, the final recommendation is to form working groups, supported by industry and regulatory bodies as well
as research funding entities. These groups should collaborate to produce a roadmap for fully
harmonized marker-based food authenticity tests, which can be implemented by various end users.
References


Diamandis, E. P. (2012). The failure of protein cancer biomarkers to reach the clinic: why, and what can be done to address the problem? *Bmc Medicine, 10*, Article 87. https://doi.org/10.1186/1741-7015-10-87


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Highlights

- A harmonized approach to validate food authenticity markers is needed
- A standardized nomenclature for food authenticity markers is proposed
- Guidelines for marker discovery, validation, and accreditation are proposed