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of the Food Safety Authority of Ireland

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The suitability of analytical methods for assessing food authenticity

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The Exchange, George's Dock, IFSC,
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T +353 1 817 1300
E info@fsai.ie

www.fsai.ie

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List of Abbreviations

Abbreviation	Term
CAC	Codex Alimentarius Commission
CI	Confidence Interval
CRM	Certified Reference Material
Da	Dalton
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
GC–MS	Gas Chromatography–Mass Spectrometry
HPLC	High-Performance Liquid Chromatography
IR	Infrared Radiation
ISO	International Organization for Standardization
LC	Liquid Chromatography
LC–MS	Liquid Chromatography–Mass Spectrometry
MS	Mass Spectrometry
MU	Measurement Uncertainty
PCR	Polymerase Chain Reaction
PDO	Protected Designation of Origin
PGI	Protected Geographical Indication
QC	Quality Control
UV	Ultraviolet

Glossary

Term	Definition
Absorbance	A measure of the quantity of light that is absorbed by a sample
Accredited laboratory	A laboratory that has acquired accreditation to a particular standard following an assessment by a national accreditation body, for example in the context of food safety, the relevant standard is EN/ISO 17025.
Analyte	The sample constituent of interest in an analysis
Bias	An estimate of a systematic measurement error
Calibration	The process of establishing a relationship between two properties
Calibration curve	A graphical representation of a calibration
Certified reference material	Reference material that is accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceability, using valid procedures
Error	Measured quantity value minus a reference quantity value
Food authenticity	The accurate representation of a food product's origin, ingredients, and quality, ensuring that it meets established standards and claims
Food fraud	The deliberate misrepresentation or deceptive labelling of food products for economic gain
Sample matrix	The components of a sample other than the analyte of interest
Mean	The average value of a set of numbers
Measurand	A quantity that is intended to be measured
Non-standardised method	A test method for which a set of protocols and procedures have not yet been established and agreed upon by the scientific community, and which has not been subjected to a collaborative study
QC standard	A quality control standard that allows users to verify their total analytical system and estimate or eliminate bias from analytical results
Validation	Confirmation, through the provision of objective evidence, that the required method performance characteristics for a specific intended use or application have been fulfilled

Executive summary

The Food Safety Authority of Ireland (FSAI) requested its Scientific Committee to advise on the criteria and analytical controls that should be considered when examining the potential use of new laboratory techniques in assessing food authenticity. A subcommittee of experts in various analytical fields was established to prepare draft advice on the most relevant analytical techniques that have or could have a role in the assessment of food authenticity, within or outside the realm of routine official controls. This draft advice was then considered by the Scientific Committee of FSAI.

Many analytical techniques are routinely used in various research and development settings and have been the subject of peer-reviewed publications, which indicates that they have a sound scientific basis. However, the successful application of laboratory analysis in a regulatory or legal context is dependent on a high level of rigour that requires both the analytical techniques and laboratories to be accredited. In the absence of accredited test methods, regulators may occasionally need to avail of unaccredited laboratory analysis for the purposes of assessing food authenticity. Anomalous analytical results, if identified, may inform decisions on further action or evaluation under official controls, including further laboratory analysis, traceability checks, audits, or inspections.

This report outlines some of the analytical techniques currently available and which may be of use in assessing the authenticity of food. The report indicates specific or general analytical controls and criteria that are required to ensure that the results obtained are reliable and reproducible. The report also emphasises that to be fit for purpose, laboratory techniques and their application must incorporate various critical controls at the sampling, processing, and analysis stages. An important control, where feasible, is the accurate analysis and reporting of unlabelled samples, whereby the precise content of those samples is not known in advance by the preferred laboratory performing the testing.

A high level of association and communication with analytical experts in the various laboratory settings supports the FSAI in ensuring that it is aware of and has access to the newest and most sophisticated laboratory techniques that could be of use in assessing the authenticity of food. Two key questions that were addressed by the Scientific Committee and their outcomes are summarised here:

What are the essential criteria (e.g. specificity, linearity, range, accuracy, reproducibility, precision, etc.) for unaccredited analytical techniques to be acceptable and reliable tools in examining the food chain, particularly in assessing food authenticity?

For an analytical method that is not based on a standardised protocol or accredited method to be considered acceptable and reliable in examining the food chain, there are a number of important issues to consider as outlined in points (i) to (vi) below. The method should, where possible, be applied in a laboratory that has a robust quality management system in place to ensure traceability and consistent performance of the method.

Important points for FSAI to consider when commissioning a laboratory to carry out non-standardised analytical testing, or when reviewing results obtained with an unaccredited, non-routine analytical technique or test method:

(i) Sample(s) should be selected, collected, stored, and transported in a manner that maintains security, traceability, and physical integrity, and prevents any adverse impact on the reliability of the laboratory analysis or results.
(ii) Representative samples from different batches of the food under consideration are preferable and should be of sufficient size to allow for replicate analyses.
(iii) Sample preparation and extraction protocols (where required) should be clearly documented and optimised for the sample matrix and analysis involved.
(iv) The laboratory should be able to provide evidence of the reliability, accuracy, precision, repeatability, and reproducibility of the method, including information on any potential effect on analytical results due to the nature or stability of the sample.
(v) The FSAI should assess the performance and reliability of the non-standardised analytical method and the competence of the laboratory, for example by submitting for analysis samples where the provenance and composition is unknown to the testing laboratory.
(vi) Where necessary and feasible, analytical results obtained using non-standardised test methods should be supported by other means, including official inspections involving traceability determination and where possible duplicate analysis using standardised/accredited test methods in a separate laboratory.

For the different categories of analytical tests (e.g. spectroscopic, molecular, omics etc.), what are the analytical controls feasible or necessary to ensure that methods are fit for purpose and results are accurate, reliable, and reproducible?

All such analyses should include the following in each batch of tests:

- System suitability check(s)
- Calibration curve where applicable

- Certified reference materials (CRMs) where these are available
- Suitable controls that can serve to answer any questions about the precision or accuracy of the test
- A suitable number of replicate analyses of test or quality control (QC) samples to confirm the reliability and reproducibility of qualitative or quantitative results produced by the method
- Results should be statistically analysed where appropriate in order to interpret the data in a meaningful way.

1.0 Introduction

1.1 Background

The pathways of food through production, processing and marketing are increasingly complex, with multiple opportunities for intentional or unintentional substitution or adulteration, including food fraud.

Food fraud is associated with the generation of unjustified financial profit at the consumer's expense. The more common forms of food fraud include substitution with cheaper and sometimes different ingredients, as witnessed during the horse meat scandal in Europe (O'Mahony, 2013), where horse meat was used to partially replace more expensive beef ingredients in a range of composite products. More subtle forms of food fraud can include the misuse of particular indicators of a food's origin or production method. For example, certain food products command a premium price based on their geographical origin (Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Speciality Guaranteed (TSG)), or the production system (organic, free range, or grass-fed) (Henchion *et al.*, 2014; Daley *et al.*, 2010, Camin *et al.*, 2017). Some consumers are willing to pay a premium for such products, and more may do so in the future as sustainability issues are set to play an increasing role in food choices (McCluskey *et al.*, 2005, Li and Kallas, 2021).

In the years since the Food Safety Authority of Ireland (FSAI) used deoxyribonucleic acid (DNA) analysis to expose the adulteration of beef products with horse meat in 2012, there has been a significant increase in the development of analytical methods designed to provide the food industry with new and more sophisticated methods of detecting and/or quantifying various substances or ingredients in food. Many of the newly developed or refined analytical methods originated in academic or other research institutions and may not have been subjected to the same level of characterisation and rigour as analytical methods used by official control laboratories (e.g. validation, accreditation, ring trials, etc.). However, these methods may be useful as screening tools in assessing food authenticity, particularly in circumstances where there are no established or standardised methods. These new analytical methods are often presented as cost-effective ways to help protect small- to medium-sized food businesses from food fraud. Food business operators using novel analytical methods should be aware, however, that the results of such testing may not provide an effective defence when official control testing of the same food identifies a regulatory non-compliance.

Accredited test methods are a legal requirement for official controls on food within the European Union (EU). Unaccredited test methods, particularly those that use analytical techniques more commonly used in research studies, could be used for screening purposes if they are subsequently

followed up by official controls where appropriate. While new test methods are usually published in peer-reviewed scientific journals, they may not meet the stringent requirements for test methods used in official food controls as set out in EU legislation and guidance. It is therefore necessary to be able to identify and assess the fitness for purpose of unaccredited analytical methods that could be employed by the food industry or regulatory authorities to detect and/or quantify anomalies in the food chain. From a regulatory perspective, it is important that any possible anomalies uncovered by new and/or unaccredited analytical methods should be subjected to further critical assessment, such as another analytical process, inspection, or documentary checks, as part of food traceability requirements.

1.2 Aim

This report has been prepared in response to a request for advice (Appendix 1) from the FSAI to its Scientific Committee. It identifies a range of analytical techniques that, when performed by expert laboratories incorporating suitable controls, could identify anomalies within a food which may help to determine the authenticity of that food.

1.3 Scope

This report identifies analytical techniques that could be used for the assessment of food authenticity. Certain analytical techniques are well established and already play a role in EU official controls for particular food categories (e.g. high-performance liquid chromatography–mass spectrometry (HPLC–MS)). Others are mainly used by academic or research laboratories and have not yet been applied as part of official controls for food authenticity purposes.

For the different categories of selected analytical methods (e.g. chromatographic, spectroscopic, molecular, etc.), sampling and analytical controls are necessary to ensure they are fit for purpose and that results are accurate, reliable, and reproducible. The analytical techniques discussed in this report should only be considered where an expert laboratory with an appropriate quality management system has been identified. Discussions with a potential laboratory should include the suitability of a technique for specific food samples, along with the controls required to ensure that the results are reliable.

1.4 Relevant EU food law

1.4.1 Official controls

The Official Controls Regulation (EU) 2017/625 (OCR) details the controls and other official activities to be performed by Member States to ensure the appropriate implementation of EU food and feed law. It defines the rules for the designation of laboratories that carry out analyses, tests or diagnoses for official controls and the conditions each one must meet to be designated as an official laboratory. Accreditation to EN ISO/IEC 17025 is mandatory for the designation of official control laboratories which must employ accredited methods when testing samples taken as part of official controls. Accreditation represents independent external assessment that a laboratory has an acceptable quality management system in place and has the ability and competence to provide reliable test results in line with the requirements of this standard.

1.4.2 General labelling legislation

Legislation on the provision of food information to consumers (Regulation (EU) No 1169/2011) is designed to ensure informed consumer choice by providing reliable and consistent food information. One of the basic tenets of this legislation relates to the requirement that “Food information shall not be misleading” (Article 7.1).

1.4.3 General principles and requirements of food law

EU legislation (Regulation (EC) No 178/2002) laying down the general principles and requirements of food law stipulates that “food shall not be placed on the market if unsafe” (Article 14). It applies to all stages of production, processing and distribution of food and feed, but not to primary production or private domestic use.

1.5 Assessing the weight of evidence

Weight of evidence is a relatively broad term which, in the context of food authenticity investigations typically encompasses the amalgamation of data and weighing of evidence during an investigation to arrive at a conclusion on the authenticity of a food product. In November 2023, The Authenticity Methods Working Group of the UK Department of Environment, Food and Rural Affairs produced a report (Defra, 2023) on a toolkit to support weight of evidence approaches for food authenticity investigations. Guidance is provided on appropriate means to assess the

authenticity of a food and/or beverage based on information acquired from different sources, including from the product's supply chain traceability and through analytical evidence. The report advises on six key overarching pre-requisites that may impact the results and strength of proof, with variables relating to the following:

(i) Sample
(ii) Quality considerations
(iii) Methodology or methodologies used
(iv) Staff experience
(v) Interpretation of results
(vi) Analysis.

2.0 Codex and Eurachem guidance on method criteria and analytical controls

Guidelines on quality assurance and essential criteria for the validation of methods of analysis have been published by the Codex Alimentarius Commission and Eurachem. These internationally recognised and accepted guidelines provide more details on the different criteria used to validate analytical methods and should be referred to if further guidance is needed on any of the criteria described in this document.

2.1 Codex Alimentarius

The Codex Alimentarius, or 'Food Code', is a collection of international standards, guidelines and codes of practice published jointly by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) to protect the health of consumers and ensure fair practices in the food trade. Codex standards are used worldwide to harmonise national food safety regulations and are recognised in the World Trade Organization Agreement on the Application of Sanitary and Phytosanitary (SPS) Measures as the international reference point underpinning trade in safe food (FAO, 2023).

The Codex Alimentarius Commission Procedural Manual includes a section on the 'Principles for the Establishment of Codex Methods of Analysis' to be used for food testing that defines the different types of methods of analysis and outlines the general criteria for their selection. It includes information on the selection of appropriate methods based on a 'Criteria Approach' which ensures that methods chosen for a particular task are fit for purpose. It also provides general criteria for the selection of single laboratory validated methods of analysis. The current version of the Procedural Manual is the 28th edition (FAO and WHO, 2023). In 2013, Codex published Guidelines on Performance Criteria and Validation of Methods for Detection, Identification and Quantification of Specific DNA Sequences and Specific Proteins in Foods (CAC/GL 74-2010).

Further explanation of essential criteria can be found in the Guidelines on Analytical Terminology (CAC/GL 72-2009), and information on applying uncertainty of measurement information is available in the Guidelines on Measurement Uncertainty (CXG 54-2021).

2.2 Codex-recommended list of analytical methods

There are many recommended methods of analysis and sampling contained in Codex standard CXS 234-1999. The list of methods is updated after each session of the Codex Committee on Methods of Analysis and Sampling. Part A of the standard lists the methods of analysis in alphabetical order of commodity category and name. This list contains information on commodity type, standard provision, method of analysis, and principle of analysis. The various commodity groups include:

- Cereals, pulses, legumes, and derived products
- Cocoa products and chocolate
- Fats and oils and related products
- Fish and fishery products
- Foods for special dietary uses
- Fruit juices
- Milk and milk products
- Natural mineral waters
- Processed fruits and vegetables
- Processed meat and poultry products and soups and broths
- Quick-frozen fruits and vegetables
- Spices and culinary herbs
- Sugars and honey
- Miscellaneous products.

2.3 Eurachem

Eurachem is a network of organisations in Europe which has the objective of establishing a system for the international traceability of chemical measurements and the promotion of good quality practices. Eurachem has also produced several useful guidance documents including:

- Terminology in Analytical Measurement: Introduction to VIM 3 (Barwick, 2023)
- Use of Uncertainty in Compliance: The Eurachem Measurement Uncertainty and Traceability Working Group (Eurachem, 2021)
- The Fitness for Purpose of Analytical Methods (Magnusson and Örnemark (eds.), 2014)
- The Selection and Use of Reference Materials (Eurachem, 2002)
- Quality Assurance for Research and Development and Non-routine Analysis (Eurachem, 1998).

3.0 Validation criteria

Validation of an analytical method is carried out to demonstrate that it is suitable for its intended purpose. Table 1 identifies certain validation requirements to be considered for analytical methods employed in food authenticity studies. The following validation criteria relate primarily to the chromatographic, spectrometric, and spectroscopic techniques discussed later, but may be applicable to other laboratory techniques also. These definitions are based on Codex and Eurachem guidelines on analytical terminology referenced in Section 2 of this report.

Table 1 Validation criteria and definitions adapted from the Codex and Eurachem guidelines on analytical terminology

Criterion	Definition
Specificity / Selectivity	Specificity/Selectivity is the ability of an analytical method to distinguish the analyte from everything else that might be in the sample. It is the extent to which the method can be used to identify a particular analyte(s) in a mixture(s) or matrix without interference from other components of similar behaviour.
Accuracy / Trueness	The accuracy of an analytical method expresses the closeness of agreement between a measured quantity value and the true quantity value of a measurand. Accuracy is influenced by both the random and systematic effects on the measurement result. Trueness is the closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value. Trueness is inversely related to systematic measurement error, which may be estimated as measurement bias, i.e. the difference between the mean value of several measurement results and a reference quantity value. Bias can be determined using Certified Reference Material (CRM) and reported as the ratio of measured and reference values, expressed as a percentage.
Precision	The precision of an analytical method is the closeness of agreement (degree of scatter) between measured quantity values obtained by replicate measurements on the same homogeneous sample under the specified conditions. The precision of an analytical method is expressed numerically using measures of imprecision such as the standard deviation calculated from results obtained by carrying out replicate measurements on a suitable material under specified conditions. Precision can be concentration-dependent and

Criterion	Definition
	should be measured at different concentrations within the working range, typically covering the lower, mid, and upper parts. Two commonly accepted sets of conditions under which precision is measured are Repeatability and Reproducibility. Repeatability refers to measurements being made on portions of the same material by a single analyst, using the same procedure, under the same operating conditions over a short time period. Reproducibility refers to measurements being made on portions of the same material by different analysts working in different locations. In collaborative method validation studies, the same measurement procedure is used at all participating laboratories.
Limit of detection (LOD)	At and above the limit of detection (LOD), a positive identification can be achieved with reasonable and/or previously determined confidence in a defined matrix using a specific analytical method. The LOD is also defined as the lowest concentration of analyte (target) that can be distinguished from the background noise with a given degree of confidence. In the case of test methods where the background signal can be measured, a minimum requirement for a signal-to-noise ratio of 3 is widely accepted. The LOD is not a robust or rugged parameter and can be affected by minor changes in the analytical system (e.g. temperature, purity of reagents, matrix effects, or instrument conditions). It is therefore important that this parameter is always verified by laboratories introducing methods validated elsewhere.
Limit of quantification (LOQ)	The limit of quantitation (LOQ) is the lowest concentration of analyte in a sample which can be reliably determined with suitable precision and accuracy. In the case of methods where the background signal can be measured, a minimum requirement for a signal-to-noise ratio of 10 is widely accepted.
Limit of reporting (LOR)	LOR is the lowest amount of analyte that a given method can routinely measure with reasonable accuracy and precision.
Linearity	The linearity of a method of analysis is its ability, within a certain range, to provide an instrumental response or results proportional to the quantity of analyte in the laboratory sample.
Range	The ‘working range’ or ‘measurement range’ of an analytical method is predefined by the purpose of the method and may reflect only a part of the full

Criterion	Definition
	linear range. Acceptance criteria usually involve a 'goodness of fit' test, often using a high correlation coefficient (r) of 0.995 as the criterion for linearity.
Robustness and ruggedness	The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, which provides an indication of its reliability during normal usage.
Recovery	Recovery is the proportion of the amount of analyte, present in or added to the analytical portion of the test material, which is presented for measurement. When using certified matrix reference materials to assess the recovery of a method, the recovery is the ratio of the concentration of analyte found to that stated to be present. Recovery is primarily intended for use in methods that rely on transferring the analyte from a complex matrix into a simpler solution, during which loss of analyte can be anticipated. In such cases, it may be necessary to apply a recovery correction factor when reporting results.
Stability	Validation of an analytical method should include tests to determine the stability of analytes in the sample matrix, in extracts at different stages of sample preparation, and in the final extract ready for analysis.
Measurement Uncertainty (MU)	Measurement Uncertainty provides a quantitative indication of the quality of a measurement result. Metrologically, MU is defined as a parameter associated with the result of a measurement that characterises the range of values that could reasonably be attributed to the analyte being measured. Any measurement that is made can have some uncertainty associated with it, and the uncertainty interval which is quoted will be the range within which the true value lies at a certain level of confidence. Typically, a 95% confidence interval is used. Testing laboratories should be able to estimate the MU for all results reported. The uncertainty is calculated by estimating the potential errors associated with the various stages of the analysis, e.g., pre-analytical effects, homogenisation, weighing, pipetting, injection, extraction, derivatisation, recovery, and preparation of calibration curves. Validation data, such as accuracy and precision under repeatability/reproducibility conditions, already account for many of these factors and should be used when estimating MU.

3.1 General considerations for laboratory analysis

- The relevant food authenticity issue(s) in question should be discussed with a potentially suitable laboratory (or laboratories) to determine whether they can offer a viable analytical solution. It is advisable where possible to include analytical experts, external to the FSAI, in conversations with potentially suitable laboratories.
- All of the analytical techniques discussed in this report are specialised laboratory-based tools and only laboratories with suitable accreditation, experience, and robust quality assurance processes should be considered.
- National or international standards relating to the analytical technique to be used and/or the reporting of results should adhered to, where they exist.
- Profiles and test sample results should be challenged to confirm the suitability and fitness for purpose of the test method or results (e.g. unlabelled samples of known provenance to be tested by the laboratory).
- The characteristics of a test method for determining the authenticity of a food or ingredient, as determined by validation or otherwise, should indicate whether it provides definitive information, or if it is useful only as a screening mechanism that provides preliminary results to be clarified or confirmed by other information (e.g. further analysis or traceability checks).

4.0 Quality control

Quality control (QC) samples are used to confirm that the analytical methods carried out by a particular laboratory at a given time are performing as expected (i.e. the test method is in control). They are used to assess method performance and identify any potential anomalies during the testing process. The following controls relate primarily to the chromatographic, spectrometric, and spectroscopic techniques discussed later, but may also be applicable to other laboratory techniques.

4.1 Control samples

The inclusion of suitable QC samples in each series of analysis is an essential part of ensuring the quality of test results. In-house QC samples can be blank or positive control samples. A blank represents the reagents used in the analysis or the matrix of the test samples but without the analyte(s) of interest. Positive controls are analytical samples which contain a known amount of target analyte(s), and which can be used to demonstrate that analytical results are specific to the target analyte(s) in question rather than potential endogenous or introduced contaminants.

Reagent blank

A control sample that is composed of the reagents used in the analysis excluding the matrix and the analyte of interest, which serves to identify any background signals or impurities that may be introduced by the reagents, equipment, or environment during the analytical process.

Matrix blank or negative sample

A matrix-matched control sample that does not contain the analyte of interest or contains it at a concentration below the detection limit of the assay, which can be used to evaluate the specificity of the assay and to confirm that the test does not produce false-positive results.

Positive sample or spiked sample

A control sample containing a known concentration of the analyte(s) of interest that can be used to validate the accuracy and sensitivity of the assay. Such samples are also used during routine analysis to provide evidence of unchanged method performance or to detect issues arising during the analysis, and to prevent erroneous results from being reported. Matrix-matched certified reference materials are the desirable option, but they may not always be available. Alternative options are spiked samples, which are blank matrix samples to which a known amount of target analyte has been added, or in-house QC samples that have been characterised by other means, such as previously analysed proficiency testing samples.

4.2 Reference materials

According to ISO/Guide 30:2015, a reference material is sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process. Uses may include the calibration of a measurement system, assessment of a measurement procedure, assigning values to other materials, and QC. A CRM is characterised by a metrologically valid procedure and accompanied by a certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability.

The key issues to consider in terms of reference materials are as follows:

- Reference materials should be of a high purity and identical to the substance(s) under investigation with documented traceability to certified international standard material. ISO 17034:2016 sets out the requirements in accordance with which reference materials, including CRMs, are produced and the general requirements for the competence and consistent operation of reference material producers.
- Reference materials should be stored in conditions recommended by the supplier.
- Preparing and dispensing reference materials should be done with appropriate equipment and materials.
- A minimum of three different concentrations of reference materials (low, mid-range and high) across the reportable range of the assay is required. The lowest standard level should be close to the minimum quantifiable point of the measurement range and below the expected concentration of any unknown sample.

4.3 Analytical standards

In the case of certain biological analyses such as DNA analysis, analytical standards consist of a specific sequence of DNA that has been shown by an extensive set of analytical tests to be authentic and of high purity. Such standards can be obtained from biological materials or artificially synthesised and should be of known concentration.

Similarly, analytical standards used for chemical analysis are produced as a high-purity reference material in solid or liquid form with a known concentration. Analytical standards are typically accompanied by a certificate of analysis outlining the product name and its purity, Confirmation of Acceptance for Studies (CAS) number, molecular weight, expiry date, chemical structure, physical properties, concentration, and related uncertainty. Where liquid-based assays are performed, the

dilution of standard material should be carried out in suitable solvents that match those in the extraction and dilution of the unknown sample being tested. Sufficient time should be allowed for obtaining new batches of analytical standards in advance of required use, which will ensure that the new standards can be validated in a series of assays run in parallel with the existing standard material to ensure comparability.

4.4 Calibration curve

Calibration is an operation that, under specified conditions, establishes a relationship between the values provided by measurement standards and corresponding indications (e.g. instrument signal) and uses this information to establish a relationship for obtaining a measurement result from an indication. For quantitative analysis, a calibration curve should be included in each analytical series. Calibration curves should consist of an appropriate number of levels, typically a minimum of five (including a blank level). In the case of semi-quantitative determinations, e.g., for screening purposes, the number of calibration points can be reduced.

5.0 Sample management

The way in which samples are collected, handled, stored, prepared, and managed in general can have an impact on the integrity and reliability of the subsequent laboratory analysis.

5.1 Representative test samples

It is important that analytical results are representative of the food that has been sampled. To ensure representative analytical results, it may be necessary to make the sample more homogenous (e.g. drying, grinding, blending, mixing, or tumbling), with multiple sample aliquots taken from different sections of a food product.

5.2 Sample storage

Sample storage conditions (e.g. suitable container, temperature, humidity, light etc.) should be a consideration due to the potential for sample deterioration to yield misleading results. The addition of preservatives (e.g. sodium azide, ascorbic acid, EDTA, etc.) to prevent sample deterioration prior to testing may be an option in certain situations where the food matrix (e.g. liquid or moist biological food material) provides a suitable environment for physico-chemical or microbiological deterioration.

5.3 Sample preparation

Sample preparation includes the process of extracting and/or purifying the analyte(s) of interest from a particular sample matrix. The sample matrix is the type and/or form of a food (e.g. plant material, simple or complex foods, or liquid or solid foods). Sample preparation is a vital pre-requisite to achieving reliable and reproducible results with many types of analysis, while minimising any adverse effects on the instrument's performance or lifespan. For some biological materials, drying prior to grinding can be an effective step to inhibit or limit endogenous processes that could negatively affect the integrity of the target analyte (Krakowska-Sieprawska *et al.*, 2022).

The selection of an appropriate extraction technique will depend on the type of analysis to be carried out, the sample matrix, and the analyte(s) of interest, among other factors. Solid-phase extraction or other fractionation techniques are the method of choice for the purification of samples prior to component separation by chromatographic techniques. Such methods are more efficient

than other extraction methodologies in reducing the number of extraction steps required and the amount of sample required for analysis.

Enzymes or combinations of enzymes with specific hydrolytic properties can be used to break up certain complex biological sample materials, thereby releasing target analytes prior to extraction.

6.0 Separation and analysis of food components

Foods are generally multi-constituent matrices made up of proteins, fats, carbohydrates, vitamins, minerals and so on. Separation of these individual components or groups of components from each other may be required to determine a profile specific to a certain food or type of food, or to determine the presence or absence of a particular target analyte.

6.1 Chromatographic separation techniques

Chromatography is a technique used to separate a mixture into its individual or groups of constituents based on partition, adsorption, size and/or charge. Depending on the type of chromatography used and the detection method employed, the various components of a complex mixture can be identified and quantified. Targeted chromatographic methods can separate one or more characteristic analytes from other components in a sample, thus allowing them to be identified directly or by comparison with standards or reference materials. Chromatography is deemed suitable on the basis of adequate chromatographic retention, peak shape, and resolution. Peaks should be eluted with a retention of at least twice the column dead volume, should be sharp and symmetrical (ideally with a symmetry factor of 1.0), and should be resolved from interferences with a resolution of ≥2.0.

Untargeted chromatographic methods generate profiles of sample constituents that can be compared with profiles from known samples to identify differences. Untargeted approaches can employ less rigorous sample preparation processes, thereby leaving more matrix components with which to build profiles (Amaral, 2021).

6.1.1 Gas chromatography

Gas chromatography (GC) is a commonly used analytical technique for separating volatile compounds that can be heated to high temperatures without significant degradation. The technique is based on the differential partitioning of sample components between a stationary phase (column) and a mobile phase (carrier gas). Components of a mixture eluting from the column are monitored by a suitable detector system. GC can be used to identify and quantify volatile organic compounds, flavour compounds and aroma compounds, for example in the classification of oils and fatty acids in foods like pastry products to determine the presence or absence of palm oil. GC instrumentation is combined with detection techniques to facilitate the identification and quantification of analytes of interest. A GC system contains four main components:

(i) The gas mobile phase (carrier gas)
(ii) The sample injection unit, which heats the liquid sample and vaporises it
(iii) The stationary phase (column), which is used to separate compounds
(iv) The detector, which detects the compounds and outputs their concentrations as electrical signals.

6.1.2 Liquid chromatography

Liquid chromatography (LC), or high-performance liquid chromatography (HPLC), is a powerful separation technique that employs a liquid mobile phase moving through a solid stationary phase to separate dissolved sample constituents based on their affinities to the mobile phase and the stationary phase. The separated components are detected and measured upon elution from the stationary phase by a suitable detector system. HPLC is commonly used for food and beverage analysis and depending on the matrix and constituents to be measured or identified, the most effective combination of stationary and mobile phases will be chosen. The predominant mode of separation is reversed-phase chromatography where a polar mobile phase, such as water and a polar organic solvent, is used with a non-polar, hydrophobic stationary phase. This mode is suitable for the analysis of drugs, toxins, vitamins, and additives in food samples. Ion chromatography is commonly used to separate highly polar ionic molecules, such as amino acids and sugars, which require more specialised stationary phases to achieve satisfactory separation. The use of ultra-HPLC methodology enables high-throughput analytical separations, 5 to 10 times faster than conventional LC methodologies, with equivalent or improved chromatographic resolution.

6.1.3 Supercritical fluid chromatography

Under defined temperature and pressure, a substance can become a supercritical fluid (SF), which exhibits physical properties that are intermediate between those of the liquid and gas phases. As pressure and temperature are increased, the density and the dissolving power of the SF increases. Supercritical fluid chromatography (SFC) is similar to normal phase chromatography, with the non-polar analytes eluting first followed by the polar analytes. Carbon dioxide (CO_2) is the most common supercritical fluid and is often modified with ethanol to elute more polar analytes.

Numerous publications (Bamba *et al.*, 2012; Montañés and Tallon, 2018; Liu *et al.*, 2020) have reported the application of SFC for the analysis of food components (e.g. triglycerides/lipids) with improved separation efficiency compared to HPLC. SFC has experienced a resurgence since the

development of UHPLC systems, which have been modified to allow for the separation of polar and non-polar vitamins in a single analysis.

6.1.4 Capillary electrophoresis

Capillary electrophoresis (CE) is a method that separates compounds based on their charge and frictional forces. The analytes are eluted and separated in a buffered mobile phase under the influence of an electrical field. A significant application of CE is in the separation of identical molecules or ions that coexist as stereoisomers or mirror images of each other (chiral separations). Owing to the diversity of techniques offered by CE, it can be used to determine the contents of both the high molecular weight compounds, such as proteins or DNA fragments, and low molecular weight compounds, such as amino acids, carbohydrates, vitamins, flavonoids, inorganic ions, and organic acids (Font *et al.*, 2008).

6.1.5 Ion mobility separation

Ion mobility separation (IMS) relies on the gas-phase ion mobility of a compound which exploits characteristics including charge, size, and shape. Separation is achieved in milliseconds, and the method can be effective with or without a chromatographic separation. This method can be incorporated into hand-held screening and fingerprinting methods, or alternatively it can be used to increase the throughput, sensitivity, and resolving power of existing LC–MS and GC–MS methods. A review by Te Brinke *et al*. (2022) summarises a number of publications on the application of IMS in measuring volatile organic compounds in olive oil and honey, and phenolic compounds in red wine.

6.2 Spectrometric techniques

6.2.1 Optical spectrometry

Optical spectrometry measures the interactions between light and physical matter. The study of spectrometry dates back to the 1600s, when Isaac Newton discovered that focusing a light through glass splits it into the different colours of the rainbow - the spectrum of visible light.

A spectrometer is a scientific instrument used to separate and measure spectral components of a physical phenomenon. It can measure the wavelength and frequency of light and facilitate the identification of the atoms in a sample. A recent study using a spectrometer and machine learning

techniques assessed the quality and purity of olive oil, demonstrating the technology's potential for assessing food authenticity (Asharindavida *et al.*, 2023).

6.2.2 Mass spectrometry

Mass spectrometry (MS) is used to separate ionised particles such as atoms, molecules and clusters based on their mass-to-charge ratio. It can be used to determine the molecular weight of the particles, and the results are presented as a mass spectrum, a plot of intensity as a function of the mass-to-charge ratio. Mass spectrometry is a very sensitive, confirmatory technique that can be applied to pure samples as well as complex mixtures. Different types of mass spectrometry detection platforms exist (single quadrupole, triple quadrupole, time-of- flight (TOF), quadrupole time-of-flight (QTOF) and Orbitrap). Selecting the most suitable mass spectrometry technique for a particular purpose requires expert advice based on detailed information about the type of sample matrix and constituent(s) to be analysed.

Mass spectrometry is often coupled with chromatographic techniques (e.g. LC–MS or GC–MS) for targeted and non-targeted analyses, and it is a technique used to confirm the integrity and authenticity of different foods. Tandem mass spectrometry (LC/MS/MS), which is a low-resolution MS technique, is an effective analytical technique for the structural characterisation and analysis of food products (Campmajó *et al*., 2019). When LC/MS/MS is used in selected reaction monitoring mode for targeted quantification of specific analytes, a typical criterion used to confirm the presence of an analyte is the 'ion ratio', which is the ratio of the response of two fragment ions for the test sample compared with the ratio obtained for a known reference standard for that analyte. The ratio should agree within a predefined range (typically 20–40%) of the reference standard for the analyte to be confirmed present in the test sample.

The use of liquid chromatography high-resolution mass spectrometry (LC-HRMS) and accurate mass measurements can provide more comprehensive information regarding the exact molecular mass, elemental composition, and detailed molecular structure of a given compound. The use of suitable detectors enables the simplification of sample preparation procedures and allows both screening and quantification to be performed in a single run, including targeted, suspect, and non-targeted analyses. For example, a UHPLC-HRMS (Orbitrap) was used to authenticate cranberry-based extracts by polyphenolic profiling and multivariate calibration methods (Hidalgo Serrano *et al.*, 2018). All diagnostic ions should also have a mass accuracy of 5 parts per million (PPM) or better when compared to the theoretical mass for the ions of interest, and typically 2–3 diagnostic ions are required to confirm identity. HRMS instruments are also useful for determining unknowns

when operated in full scan mode, as the data gathered can be compared to libraries of known spectra to give presumptive identification.

6.3 Spectroscopic techniques

Spectroscopy is the analysis and interpretation of electromagnetic spectra. It can yield information about structure and other properties associated with a test material, down to the level of atoms or groups of atoms (molecules) which possess discrete energy levels. The choice of spectroscopic method for a particular analysis depends on the purpose, the level of detail required and the nature of the food product/specific food matrix.

Spectroscopic techniques are generally non-destructive and require relatively low amounts of sample and sample preparation (Sanchez *et al.*, 2020; Edwards *et al.*, 2021). Spectroscopic analysis has been incorporated into portable hand-held devices (Müller-Maatsch and van Ruth, 2021), with potential for use in assessing food authenticity (Oliveira *et al.*, 2020; Silva *et al.*, 2020).

6.3.1 Ultraviolet/Visible spectroscopy

UV/Visible spectroscopy measures the interaction of ultraviolet (UV) and visible light with molecules present in a sample. Specifically, it measures the absorption or transmission of light in the UV and visible regions of the electromagnetic spectrum, which provides information about the chemical composition of the sample analysed. Some reviews highlight the use of UV/Visible spectroscopy for food quality and authenticity investigations into meat, fish, poultry, egg, dairy products, fruits and vegetables (Chaudhary *et al.*, 2022; Haque *et al*., 2021).

6.3.2 Fluorescence spectroscopy

Fluorescence spectroscopy involves the emission of light from a sample which has absorbed light of a particular wavelength. When a sample is exposed to a specific wavelength of light (the excitation wavelength), this excites the molecules within the sample, causing a temporary transition to higher energy states. After excitation, these molecules release energy in the form of light when returning to their original energy state. The emitted light (fluorescence) has a longer wavelength than the excitation light and is detected using a fluorescence spectrometer which measures the intensity of the emitted light at various wavelengths to generate a fluorescence spectrum. Fluorescence spectroscopy has been used in the analysis of fish and meat products (Hassoun *et al.*, 2019; Karoui *et al*., 2017).

6.3.3 Infrared spectroscopy

Infrared (IR) spectroscopy measures the interaction of IR radiation with the molecular vibrations of the sample under investigation by absorption, emission, or reflection. It can detect and analyse specific functional groups and chemical bonds present in food samples. Different functional groups exhibit characteristic absorption frequencies, allowing the identification of compounds such as additives, proteins, carbohydrates, and fats. Infrared spectroscopy is conducted using an IR spectrometer which produces an IR spectrum and may be useful in characterising new materials or identifying and verifying known or unknown samples. Comparing the spectral information obtained from test samples with established references can determine the authenticity or quality of a food product.

The IR portion of the electromagnetic spectrum is usually divided into three regions: near-, mid-, and far- infrared (NIR, MIR, and FIR), named for their relation to the visible spectrum. NIR is the higher energy level while FIR has low energy levels. Both NIR and MIR are suitable for routine testing and screening, quality control purposes, and to enable real-time monitoring of food production processes. Infrared spectroscopy combined with chemometric analysis may have some use in the investigation of food authenticity (Rolandelli *et al*., 2024).

In Fourier Transform IR (FTIR), IR spectra are derived indirectly from the changes in an interference pattern of the incident and emitted light waves as the detector is moved. Fourier Transformation is a statistical treatment which is used to rapidly collect and process spectral data to give much improved sensitivity compared to conventional IR spectra. Limm *et al*. (2023) used FTIR and chemometrics for the rapid screening of economically motivated adulteration of honey spiked with corn or rice syrup.

6.3.4 Raman spectroscopy

Raman spectroscopy provides a structural fingerprint by illuminating a sample with a laser and analysing the scattered light that interacts with the molecules within a sample. The scattered light is collected and directed through a spectrometer, which disperses the light into its component wavelengths, with a suitable detector recording the intensity of the scattered light at different wavelengths. This generates a Raman spectrum, which contains characteristic peaks corresponding to specific molecular bonds and functional groups present in a food sample. The use of Raman spectroscopy in the authentication of fish species has been reported (Hu *et al*. 2023).

6.3.5 Terahertz spectroscopy

Terahertz spectroscopy uses the terahertz frequency range to analyse the unique spectral characteristics of materials. It can penetrate many organic and inorganic materials (even packaging), without causing any damage or ionisation to the food. The resulting terahertz spectrum provides information about the absorption and dispersion of terahertz waves by molecules in a sample, which can then be used to characterise the sample by comparing it to established reference spectra. A method to identify green tea and accurately determine its geographical origin has been developed based on terahertz spectroscopy (Li *et al.,* 2017).

6.3.6 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) is a physical phenomenon in which nuclei in a strong constant magnetic field are perturbed by a weak oscillating magnetic field and respond by producing an electromagnetic signal with a frequency that is characteristic of the magnetic field at the nucleus. The energy absorbed by the nuclei can be detected to produce an NMR spectrum which displays peaks that correspond to different compounds within the sample. NMR is widely used to determine the structure of organic molecules in solution, and the most important applications for chemists are ^{1}H (proton) NMR and ^{13}C NMR. Proton NMR may be used to identify and quantify sugars, organic acids, amino acids, lipids, and other metabolites in foods while ^{13}C NMR may be used to detect and quantify specific markers or metabolites associated with certain food types, geographical origins, or processing methods. By comparing the NMR spectra of authentic food samples with those of potentially adulterated or counterfeit samples, differences in molecular profiles can be identified, which could indicate authenticity issues. High-resolution ^{1}H NMR has been used to assess suspect spirit samples in which results were compared to authentic products (Kuballa *et al.*, 2018).

6.3.7 Atomic absorption spectroscopy

The absorption of light by free atoms in the ground state forms the basis for atomic absorption spectroscopy. It can be used for the quantitative analysis of metals and metalloids in food samples, particularly trace elements and heavy metals that may be present at low levels.

By measuring the concentration of specific elements, atomic absorption spectroscopy can identify potential adulteration, substitution, or contamination in food samples (Dos Santos *et al*., 2012).

6.3.8 Hyperspectral imaging

This technology combines imaging and spectroscopy to capture both spatial and spectral information. It can analyse multiple wavelengths of light and generate detailed spectral profiles of food samples which can then be used to classify or authenticate food samples. Machine learning algorithms can be trained using reference data to detect specific characteristics related to quality or adulteration in foods. Hyperspectral imaging is non-destructive and non-invasive, and allows rapid screening of large quantities of food products without compromising the integrity of the food.

Hyperspectral imaging may assist in determining the provenance or quality of various foods, such as rice (Edris *et al.*, 2024) and saffron (Malavi *et al.*, 2024).

6.3.9 Laser-induced breakdown spectroscopy

Laser-induced breakdown spectroscopy (LIBS) utilises a high-energy laser pulse to generate a plasma spark on the surface of a sample. This spark produces intense light emission, and the emitted light is analysed to determine the mineral composition of the sample. When used in conjunction with machine learning tools, LIBS may have the potential to aid the real-time detection of olive oil adulteration with lower-quality oils, including pomace, soybean, sunflower, and corn oils (Nanou *et al*., 2023).

6.3.10 Energy dispersive X-ray fluorescence

Energy dispersive X-ray fluorescence (ED-XRF) is a non-destructive analytical technique used to obtain elemental composition information from a sample, based on the interaction between a source of X-ray excitation and the elements present in a sample. One advantage of using ED-XRF is that it allows for simultaneous elemental analysis, and instruments can be designed to be small and portable. Concentrations measured can range from 100% down to PPM and samples can be screened for quick identification of elemental composition with little or no preparation of the sample required. The technique has been used in hand-held screening devices.

Numerous publications have reported on the efficacy of ED-XRF as a tool that enables the discrimination of the geographical origins of different foods, including Spanish honeys with a Protected Denomination of Origin (PDO) (Ghidotti *et al.*, 2021), wheat flours (Chen *et al.*, 2021), and Italian extra virgin olive oils from different territories (Scatigno and Festa, 2021).

6.4 Inductively coupled plasma optical emission spectroscopy and inductively coupled plasma mass spectrometry

Inductively coupled plasma optical emission spectroscopy (ICP-OES) is a type of emission spectroscopy that uses the inductively coupled plasma to generate excited atoms that emit electromagnetic radiation at wavelengths characteristic of a particular element. The intensity of the emissions at the different wavelengths are proportional to the concentrations of the different elements within a sample. Inductively coupled plasma mass spectrometry (ICP-MS) is a type of mass spectrometry that uses an inductively coupled plasma to ionise the sample, and it can be used to measure elements at very low levels in food samples. Both are multi-element, high throughput, destructive techniques that require samples to be in liquid form, which means that food samples are generally digested in acid prior to analysis. Although both techniques are capable of measuring very low concentrations of elements, ICP-OES is mainly used to measure element concentrations at parts per billion (ppb) levels and ICP-MS is the preferred choice where lower element concentrations are to be measured, such as parts per trillion (ppt). ICP-MS has a wide dynamic range, which allows multiple elements at different concentrations to be determined simultaneously. Pérez-Rodríguez *et al.* (2023) used ICP-MS to measure 22 trace elements for cereal bar authentication based on trace element fingerprints and multivariate data analysis. It has also been shown that characterisation of the elemental fingerprint of food is an efficient way to authenticate its geographical origin (Gonzalvez *et al*., 2009).

Trace elemental analysis or trace elemental fingerprinting (TEF), which is based on the concentration of several chemical elements determined via ICP-MS or ICP-OES, has been used to determine the geographic origin of seafoods, including fish (Albuquerque *et al.,* 2016; Miller *et al.,* 2013; Correia *et al.*, 2012) and bivalve shellfish (Bennion *et al.*, 2019; Zhao and Zhang, 2016; Bennion *et al.*, 2021; Ricardo *et al.*, 2015; Morrison *et al.*, 2019). Research (Bennion *et al.*, 2019; Bennion *et al.*, 2021; Morrison *et al.*, 2019) has shown that TEF based on the organic tissues and the shells of shellfish can identify the harvest location of bivalve shellfish with 100% success at a very high spatial resolution, including between sites within the same bay located just 6 km apart. Additionally, TEF is reportedly able to distinguish between harvest dates just six weeks apart (Bennion *et al.*, 2021, Morrison *et al.*, 2019).

7.0 Profiling using stable isotope ratio analysis

Stable isotope ratio analysis is a powerful technique that can be used for food authentication and traceability, for example in identifying the geographic origin and adulteration of plant and animal food products. Isotopes are the atoms of a given element which have the same number of protons but have different numbers of neutrons, and which therefore have different masses. For example, carbon occurs in two stable forms: the lighter ^{12}C has 6 protons and 6 neutrons in the nucleus and thus an atomic mass of 12; the heavier ^{13}C has 6 protons and 7 neutrons with an atomic mass of 13. The properties of the nuclei of stable isotopes do not change over time and the stable isotope composition of plants and animals reflects the nutrients, feed, and water available during growth.

Stable isotope ratios are determined using a continuous flow stable isotope ratio mass spectrometer, and this equipment is coupled with an elemental analyser that determines the small differences in the abundance of heavy and light isotopes of the same element to calculate isotope ratios such as $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$. A liquid, or more typically a solid sample, is converted by high temperature into a gas which is then carried by a continuous flow of helium gas to the isotope ratio mass spectrometer. An ion beam is generated for each gas by an ion source and the element-specific beam is split into multiple beams based on the molecular weight of the various isotopes in the gas. A detector in the mass spectrometer measures the deflection of each ion beam which determines the mass-to-charge ratio of the ions and hence the isotopic composition of the sample.

The primary elemental targets of stable isotope ratio analysis in food are carbon (C), nitrogen (N), hydrogen (H), oxygen (O) and sulphur (S), which have been used in determining the authenticity of foods such as juice, wine, milk, honey, oil, and meat based on their geographic origin (Katerinopoulou *et al.,* 2020; Mai *et al.*, 2019; Kim *et al.*, 2015; Yanagi *et al.*, 2012; Gonzalvez *et al*., 2009; Kelly *et al*., 2005).

The carbon stable isotope ratios of plants differ according to the type of photosynthetic pathway used, reflecting differences in isotopic discrimination involved in carbon dioxide (CO_2) fixation during C3/C4 photosynthesis, as well as the isotope ratio of the CO_2 being fixed. The nitrogen isotope ratios in plants are largely determined by those of the nitrates and ammonia in soils.

Stable isotopes in animal tissues are largely determined by the animal's diet (Monahan *et al*., 2018). For example, beef from cattle consuming a predominantly C3 (grass-based) diet compared to that from animals consuming a predominantly C4 (maize-based) diet could be distinguished by ^{13}C analysis (Bahar *et al.*, 2005). However, stable isotope analysis of meat products may only be able to identify sources to the international (e.g. USA or EU) (Schmidt *et al.,* 2005) or regional level (Perini *et al.*, 2009).

Animal diets and the environmental growing conditions for plants may change over time and so isotope analysis on its own may not be sufficient and may require supplementation with other analytical methods, such as micro-nutrient profiling, to make a reliable determination of the origin of the food. For example, stable isotope analysis coupled with chemometrics was used to determine the geographical origin of rice (Wang *et al.*, 2020), and stable isotope analysis combined with fatty acid analyses was successfully used to determine the harvest location of squid (Gong *et al.,* 2018). In addition, stable isotopes of animal muscle and fat "turns over" so that isotopic ratios infer recent feeding conditions only and possibly not that reflecting the overall lifespan of the animal.

Analysis of oxygen isotopes has been used to determine the authenticity of seafood (Martino *et al.*, 2022) and to verify the origin of wine using carbon and oxygen isotope analysis (Wu *et al.,* 2021). However, as is the case for many meat products, the use of oxygen isoscapes (the isotopic ratio of an element over large spatial scales) to determine the origin of seafood may only yield results relating to broad-scale geographic locations, and its effectiveness declines in the case of species with wide-ranging migrations such as tuna (Martino *et al.*, 2022). Stable isotope ration analysis could be a consideration in attempts to verify the origins or contents of various foodstuffs, but it may not be a suitable tool for determining authenticity in all contexts, particularly those where other technologies such as NMR or mass spectrometry may be more effective.

8.0 Profiling using DNA analysis

8.1 Introduction

All organisms contain nucleic acids in the form of deoxyribonucleic acid (DNA) and/or ribonucleic acid (RNA). DNA consists of four nucleosides generally referred to as A (adenosine), C (cytidine), G (guanosine), and T (thymidine). The precise sequence of these four DNA nucleosides is characteristic of an organism and, except for random mutations, is not generally impacted by the developmental or growth stage of that organism. This is one reason that DNA analysis has become a reliable forensic tool in the investigation of various types of criminal activity, and more recently in assessing food authenticity.

Species determination is the main application for DNA analysis in assessing food authenticity. The FSAI has availed of DNA analysis as part of routine official controls in the detection and quantification of genetically modified (GM) foods since 2000.

8.1.1 DNA analysis

DNA is a relatively robust molecule that can persist in various environments and on many surfaces while retaining sufficient integrity to enable detection/quantification and source identification. The DNA sequences (complete and incomplete) of many organisms (plants, animals, and microorganisms) have been elucidated in recent decades and as part of the peer-reviewed publication process are usually deposited in international databases where they become publicly available. Interrogation of these databases enables the identification of the organism from which a particular DNA sequence derives, based upon the accuracy of the metadata. The main laboratory techniques involving DNA analysis for food authenticity purposes include polymerase chain reaction (PCR) and DNA sequencing. The European Network of GMO Laboratories (ENGL) has published guidance on DNA extraction methods used in the context of official controls relating to genetically modified organisms (GMOs) in food and feed (European Commission, Joint Research Centre, 2024).

8.2 Polymerase chain reaction

8.2.1 Introduction

Even when present initially in very low amounts and/or partially degraded, selected segments of DNA can be exponentially amplified using PCR to yield greater amounts that can then be further analysed.

PCR involves the repeated enzymatic generation (amplification) of a specific segment of DNA of limited size by adding individual nucleotides to short synthetic DNA fragments called primers. A certain level of knowledge about the source of the DNA in question is required to be able to select the specific DNA primers that will ensure that only a specific section of the DNA from a particular source is amplified. The DNA products of PCR amplification are routinely detected and reported in real time (real-time PCR) as the reaction proceeds. However, the DNA products of PCR can also be directly visualised following size-based separation using agarose gel electrophoresis, a method that can have benefits when communicating such results to an audience less familiar with DNA analysis.

Sample preparation is an important consideration when conducting PCR analysis. Despite the relatively robust nature of DNA, refining and processing of some foods can result in degradation of the DNA which may adversely affect the PCR reaction. An expert laboratory should be able to demonstrate that their DNA extraction process is suitable for yielding DNA of sufficient quality and quantity from the target sample to facilitate PCR analysis. The laboratory should also be able to demonstrate its competency to carry out reproducible and reliable PCR on the food(s) of interest. If quantitative PCR is performed, the laboratory's competence in that service should be demonstrated and the limits of quantification should be defined.

8.2.2 PCR controls

Each laboratory should include appropriate controls to ensure that the PCR reaction is functioning reliably to the required standards within that laboratory. The possibility of false positives and false negatives can be managed by appropriate controls and, when required, by the sequencing of amplified DNA fragments.

8.3 DNA sequencing

DNA sequencing involves elucidation of the sequence of the four nucleosides that make up DNA and can be used to confirm the identity of the source of DNA present in food. Although 'Sanger'

sequencing has been the primary DNA sequencing technology for many years and is still in use, the technically more complex method of next-generation sequencing (NGS) has become commonplace.

Figure 1 Sanger DNA sequencing illustration DNA primer Newly synthesised DNA

Sanger sequencing is limited to sequencing DNA from one source at a time and requires some prior knowledge about the DNA to be sequenced. However, NGS does not require prior knowledge of the DNA to be sequenced and DNA from multiple source organisms can be sequenced in parallel (e.g. metagenomics). Since the basic technology was initially developed, improvements in the various technical platforms continue to bring technological advantages as well as greater cost efficiency and thus affordability.

With NGS, the large amount of raw DNA sequence data generated must be processed through computer-based bioinformatics programmes by experienced operators. Aligned DNA sequences can then be used to interrogate established international DNA sequence databases to identify a possible source organism. Where NGS has been used as a screening tool to identify the possible presence of various organisms or associated material in a food, confirmation by other means may still be prudent. For this purpose, traceability documentation or additional laboratory analysis such as targeted PCR or immunological methods, including ELISA should be considered.

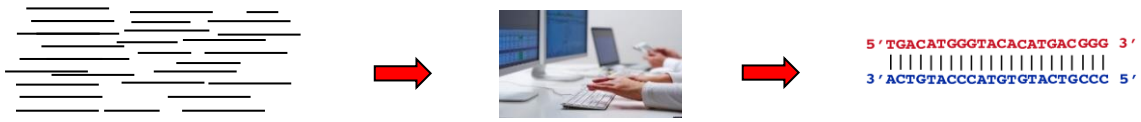

Figure 2 Next-generation sequencing illustration

NGS is central to a number of investigational methodologies availing of the information provided by DNA sequences, including the identification of specific microbial agents suspected of causing food poisoning. NGS could also play a role in identifying the bacteria in certain food supplements purporting to contain ‘probiotic bacteria’ and the presence of undeclared plant species in foods and food supplements (FSAI, 2019).

9.0 Proteomics (protein-based analysis)

9.1 Introduction

Proteomics is the large-scale analysis of proteins in a biological system at a given time (Pandey and Mann, 2000). The protein profile of an organism (or part thereof) is characteristic of that organism at a particular time and can, in certain circumstances, be used to identify material from individual organisms or parts thereof. DNA is detectable in an organism throughout its lifespan, unlike some proteins which are only transiently present in a cell in response to various internal or external stimuli. This can limit the usefulness of protein profiles in food authenticity assessment. In addition, the protein profile of various parts of a eukaryotic organism can vary depending on their specific functional roles or location. For example, the protein profile of leaf material can be different to that of the stem or roots from the same plant.

9.2 Protein-based analysis

Various approaches to studying proteins either individually or as profiles are routinely used for research and development purposes and some may, in the right circumstances, play a role in food authenticity studies. An overview of analytical protein methodologies is provided by Ortea *et al.* (2016). One basic protein profiling method is 1- or 2-dimensional SDS PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) analysis utilising a range of dyes (e.g. Coomassie blue, silver staining, etc.) to visualise the proteins separated by size and/or electric charge. However, such protein profiles are best suited to single-ingredient foods because the number of potential variables in multi-ingredient foods makes reliable profile comparisons impractical.

The use of mass spectroscopy for protein profiling is well documented and its use in food authenticity has been documented (Valletta *et al.* 2021). However, a considerable amount of preparatory work is required in order to establish a usable database of proteins for a particular food product. Many other protein analysis tools are utilised in research-based environments, but their effectiveness in assessing the authenticity of food has yet to be established. It may also be difficult to identify laboratories able to carry out such sophisticated analysis.

9.3 Immunological analysis of proteins

Antibodies form part of the body's natural defence system in response to invading organisms such as bacteria and viruses, and other foreign material. Antibodies generally interact with, and bind only to, the proteins or parts of the proteins (epitopes) that elicited their production, although

interaction with similar but unrelated proteins can also occur (cross-reactivity). Antibody-protein specificity can be exploited in laboratory methods to detect the presence of certain proteins in multi-ingredient foods. For example, most national authorities in the EU use antibodies raised in animals against proteins from known allergenic foods to detect the undeclared presence of those allergenic foods (e.g. peanuts, milk, and egg) in multi-ingredient foods. The analytical method of choice is ELISA, which is further discussed under Protein Binding Assays in Section 10.4. Antibodies can also be raised against organ-specific proteins in various food animals and at least one commercial company in the UK offers antibody-related testing that can distinguish between bovine offal and meat in food.

The risk of cross-reactivity with similar but unrelated proteins should be clarified with the laboratory before using immunological methods for authenticity assessment to ensure that the risk of false positives is understood and managed.

10.0 Profiling using bioassay methods

10.1 Introduction

Bioassay methods include a wide variety of measurement applications that use biological constructs such as enzymes, antibodies, whole cells, or whole organisms (e.g. microbes) as tools to detect or quantify specific compounds in food products.

10.2 Hazard identification bioassays

Several types of bioassays are used to assess contact exposures from toxic substances such as migration of food contact and packaging plastics, or dioxins, etc. in food samples. The bioassay endpoints can be a measurement of cytotoxicity, genotoxicity, or endocrine disruption (Severin *et al.,* 2017), as outlined in Table 2. The detection methods may be straightforward, such as measurement of turbidity at standard wavelengths or measurement of luminescence or fluorescence following the addition of fluorescently labelled or luminous dyes (e.g. Fluorescein isothiocyanate and luminol).

Table 2 Bioassay methods (adapted from Severin *et al.* 2017)

Bioassay type	Biological model	Outcome principle	Measurement
Cytoxicity	Mammalian cell lines	Loss of cell permeability	Trypan blue exclusion
Genotoxicity	Bacterial and mammalian cells	Altered mutation rates, DNA strand breaks, etc.	Comet assay, Rec assay, Ames test, micronucleus test
Endocrine disruption	Human cell lines, yeast cell lines with stably integrated human estrogen or androgen receptors	Alteration of estrogen-responsive genes	Reporter gene assays, e.g. stably transfected transactivation (STTA) assay

10.3 Microbiological growth assays

Some micronutrients are present in very low quantities in certain food products, and this can pose sensitivity challenges for some detection methods. Pre-analytical clean-up methods can cause loss or destruction of active species or fractions. As a result, microbiological growth assays continue to be used. Some microbiological assays are extremely sensitive, reaching limits of detection that are not achievable by other techniques. However, because they provide an indirect rather than a direct measurement of an analyte, they are gradually being replaced by newer technologies such as LC/MS/MS or GC/MS/MS (Höller *et al.*, 2018). For example, Okamoto *et al.* (2021) assessed the vitamin B_{12} content of commercially available edible insect products using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS).

10.4 Protein-binding assays

Protein-binding assays are based on methodologies in which the analyte of interest competes with an identical test-based 'labelled' analyte for attachment to a protein that has high affinity for the analyte. The chemistry relies on steady-state conditions being established between labelled and unlabelled analytes, as well as the provision of and an equal affinity of the protein for the labelled and unlabelled analytes in calibrators and in specimens of unknown concentration. The test is carried out with fixed concentrations of labelled analyte and high-affinity protein. Both competitive and non-competitive binding assays are established formats. The inverse relationship between the amount of labelled analyte bound to the protein in the presence of competing unlabelled standard or unknown concentrations is used to create calibration curves, calculated in terms of the percentage of bound/unbound labelled analyte and used to determine the concentrations of unknowns.

The current protein-binding assays include ELISA, Fluorescence Immunoassays and chemiluminescence immunoassays. Newer generation assays tend to use monoclonal antibodies as the binding protein in order to optimise affinity and specificity. However, not all protein-binding assays are immunoassays, with some relying on the unique specificity and high affinity of a compound for its biological carrier protein (e.g. vitamin B_{12} and intrinsic factor). Currently, immunoassays and other protein-binding assays are only a minor quantitative tool in food chemistry laboratories, but the technology is also used in solid phase sample preparation steps (Nováková, 2013; Niu *et al.,* 2018). Sample preparation steps can be simplified in analytical procedures that rely on the affinity of a compound of interest for a binding protein or an antibody. The more specific a food compound is for a binding protein, the less prior sample preparation may be required.

11.0 Metabolomics (metabolite analysis)

11.1 Introduction

Metabolomics can be defined as a profiling technique used to quantitatively detect metabolites in a biological system (Bujak *et al.*, 2015; Fiehn, 2001). The metabolome is the cumulative set of metabolites, low molecular weight compounds (<1500 Daltons) that are characteristic of an organism, cell, or tissue at a given time. Metabolomic studies typically combine high-throughput analytical chemistry and multivariate data analysis to establish the metabolic signature of a biological system that can be used to check for authenticity (Bujak *et al.*, 2015) using analytical techniques such as spectroscopy or spectrometry.

11.2 Untargeted metabolomics

Untargeted metabolomics can be described as a 'hypothesis-generating discovery strategy' involving the analysis of all metabolites that are present in a sample. This approach is useful when the metabolites that distinguish one food product from another are not known. It generates a large volume of data detailing the metabolomic profile for each food analysed which can be stored in a computational repository for future study using multivariate statistical analysis. These metabolites, whose identity is then confirmed against standards or repository databases, can be used in targeted analysis as biomarkers for food authentication. The main advantages of untargeted approaches include the unbiased and comprehensive accumulation of data, a high throughput of screening, and the possible discovery of unexpected (yet informative) compounds in the samples. Some limitations associated with the application of these approaches include possible false positives or false negatives, the semi-quantitative nature of these approaches and the sometimes-difficult interpretation of data (Selamat *et al.*, 2021).

Metabolic fingerprinting: This term refers to an untargeted approach that is not motivated by any preliminary assumption but aims to detail changes within the whole metabolome that can occur at a specific time in a cell, tissue, or organism (Bujak *et al*., 2015).

11.3 Targeted metabolomics

Targeted metabolomics can be described as the analysis of characterised metabolites that have been previously identified as biomarkers for a defined food product. The main advantages of employing targeted approaches include the low limit of detection, the possibility for quantitative analyses and more direct interpretation of data and analyses. Some limitations associated with the

application of targeted metabolomic approaches include the limited number of target compounds, the need to identify a target compound prior to analysis, and the requirement for quantification to utilise standards/reference material (Selamat *et al*., 2021). Metabolic profiling and metabolic footprinting are the two key approaches employed for targeted analysis.

Metabolic profiling: A targeted approach which involves the identification and quantification of predetermined groups of metabolites with similar physicochemical properties (e.g. carbohydrates, amino acids, organic acids, nucleosides) or taking part in the same biochemical pathway (e.g. glycolysis, gluconeogenesis, beta-oxidation, or citric acid cycle) at a given time under specified conditions.

Metabolic footprinting: A targeted approach which is focused on compounds secreted or failing to be taken up by cells from specific media (i.e. the exometabolome) at a given time under specified conditions.

12.0 Conclusions

Food authenticity remains an issue that the food industry and regulators continue to manage, with some success. The global nature, length and complexity of the food chain means that there are numerous opportunities for the intentional or unintentional substitution of declared ingredients with undeclared ingredients or the addition of chemicals or other agents. A useful tool in the assessment of food authenticity in recent years has been the use of laboratory analysis on a targeted and untargeted basis. A considerable amount of routine laboratory analysis is carried out in Ireland each year by official control laboratories to detect, and where necessary quantify predetermined analytes using accredited methods. Routine official control laboratory techniques are well characterised and harmonised across the EU yielding reliable, reproducible and actionable results. Added to these laboratory analyses are regulatory audits and inspections of food businesses by national authorities to ensure that traceability measures are in place as mandated by EU food law. However, the growing complexity of the food chain and the potential for alteration or adulteration of food demands that regulators must continue to explore all available means with which to assess food authenticity.

To this end, and in addition to official controls, the FSAI has had some success using various unaccredited analytical techniques as a first step in assessing the authenticity of various foodstuffs. The availability of newer technologies and laboratory techniques more common to the research and development area adds considerably to the ability of regulators to expose anomalies in the food chain. However, the use of newer analytical techniques that have not yet been standardised and adopted by accredited laboratories, even as screening techniques, requires a certain level of rigour and controls to ensure that they are fit for purpose and can yield reliable and reproducible results. This report, and any future update, provides a guide for the FSAI and other interested stakeholders in signposting various analytical techniques and relevant controls that may prove useful in assessing food authenticity.

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Appendix 1: Request for advice

Request for advice from the Scientific Committee

Topic title: The suitability of analytical methods for food authenticity use
Date requested: October 2021
Date accepted: 8 December 2021
Target deadline for advice: End 2023
Form of advice required: An assessment of the numerous analytical techniques becoming available and their suitability for food authenticity use in Ireland/EU.

Background

Since the FSAI used DNA technology to expose the adulteration of beef products with horse meat in 2013, there has been an evident increase in the area of analytics designed to provide the food industry with new, more sophisticated, and rapid ways of detecting and/or quantifying various substances/ingredients in food. Analytical techniques are frequently used to detect and quantify substitution or adulteration in food (food fraud) but are also used by the food industry to support any compositional claims they may wish to make.

Many of the newly developed or refined analytical techniques originate in academic or other research institutions and are targeted at the food industry as efficient and cost-effective ways to ensure their food complies with food law, particularly with respect to food authenticity. However, many of these new analytical techniques are not subjected to the same level of scrutiny as analytical methods used by official control laboratories (e.g., validation, accreditation, ring trials, etc.). As a result, they can produce results that conflict with those from accredited official control laboratories operating in line with EU legislative requirements. Any regulatory non-compliance can have a detrimental effect on the normal functioning of a food business and its reputation especially if the food business has been diligent and tested the product where necessary.

Accredited test methods used by accredited laboratories are a standard requirement for official controls on food within the EU. However, certain test methods never achieve accreditation, particularly new methods more common to research roles and not routinely used in official food controls. In addition, small to medium-sized food businesses may not have sufficient resources to enable them to employ suitably accredited commercial laboratories leaving them reliant on more affordable but unaccredited laboratories using unaccredited tests.

In the new age of evolving ways of committing food fraud, all scientific methods should be available to uncover such fraud and thereby protect consumers' health and interests. While new

test methods can get published in peer-reviewed scientific journals, this on its own does not guarantee that they can compete in terms of reliability and reproducibility with the stringent requirements for test methods used in official food controls and set out in EU legislation and guidance. It is therefore necessary to be able to identify and assess the fitness for purpose of new unaccredited analytical techniques that could be employed by the food industry and by regulatory authorities to detect and quantify anomalies in the food chain.

Questions to be addressed by the Scientific Committee

1. What are the essential criteria (e.g., specificity, linearity, range, accuracy, reproducibility, precision, etc.) for analytical techniques that are not yet part of the routinely used and accredited suite of tests available to official control laboratories to be acceptable and reliable tools in examining the food chain, particularly in detecting and quantifying food authenticity?
2. For the different categories of analytical tests (e.g., spectroscopic, molecular, omics etc.), what are the analytical controls feasible or necessary to ensure that methods are fit for purpose and results are accurate, reliable, and reproducible.

Appendix 2: List of analytical techniques covering a range of food groups

Table 3 List of selected analytical techniques published in the scientific literature for food authenticity analyses with corresponding food matrices

Matrix	Problem/incident	Analytical method	Reference
Almonds (ground)	Peanut addition	Inductively coupled plasma optical emission spectroscopy (ICP-OES)	(Esteki *et al.*, 2017)
Butter	Palm or coconut oil addition	Fluorescence spectroscopy	(Dankowska *et al.*, 2014)
Cheese	Plant oil addition	Fluorescence spectroscopy	(Dankowska *et al.* , 2015)
Coffee	Arabica versus Robusta	Nuclear magnetic resonance (NMR)	(Monakhova *et al.*, 2015)
Cooking oils	Variety substitution	Fourier-Transform Infrared spectroscopy (FTIR)	(Ozulku *et al.*, 2017)
Fish	Catch area	Deoxyribonucleic acid polymerase chain reaction-random amplified polymorphic DNA (DNA PCR-RAPD)	(Behrmann *et al.*, 2015)
Fish	Species substitution	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS)	(Stahl and Schröder, 2017)
Fruit juice	Apple juice addition	High-performance liquid chromatography (HPLC)	(Spinelli *et al.*, 2016)
Fruit (plums)	Organic production and cultivar verification	Mass spectrometry (of volatiles)	(Cuevas *et al.*, 2016)
Grains	Authenticity	Near-infrared (NIR) and ultraviolet-visible (UV-Vis) spectroscopy	(Wilkes *et al.*, 2016)

Matrix	Problem/incident	Analytical method	Reference
Herbs (dried)	Authenticity	Fourier-Transform Infrared spectroscopy (FTIR) and mass spectrometry	(Black *et al.*, 2016)
Honey	Sugar addition	Nuclear magnetic resonance (NMR)	(Spiteri *et al.*, 2015)
Honey	Sugar addition	Isotope ratio mass spectrometry (MS)	(Tosun, 2013)
Honey	Floral origin	Mass spectrometry and isotope ratio mass spectrometry and Raman and near-infrared (NIR) spectroscopy and inductively coupled plasma mass spectrometry (ICP-MS)	(Jandrić *et al.*, 2015)
Margarine	Fat profile	Raman and near-infrared (NIR) spectroscopy	(Üçüncüoğlu *et al.*, 2013)
Meat	Species adulteration	Near-infrared (NIR) and ultraviolet-visible (UV-Vis) spectroscopy	(Ropodi *et al*., 2017)
Meat	Adulteration with offal	Infrared (IR) spectroscopy	(Hu *et al.*, 2017)
Meat	Species adulteration	Deoxyribonucleic acid (DNA) hybridisation probes	(Rahmati *et al*., 2016)
Milk powder	Melamine	Near-infrared (NIR) spectroscopy	(Scholl *et al.*, 2017)
Milk	Nitrogen enrichment	Mass spectrometry	(Frank *et al.*, 2017)
Milk	Additives for shelf-life extension, dilution	Infrared (IR) spectroscopy	(Botelho *et al.*, 2015)
Olive oil	Geographic origin	Mass spectrometry	(Gil-Solsona *et al.*, 2016)
Parmigiano Reggiano	Fatty acid profile	Mass spectrometry	(Caligiani *et al.*, 2016)

Matrix	Problem/incident	Analytical method	Reference
Rice	Variety mislabelling	Deoxyribonucleic acid polymerase chain reaction-random amplified polymorphic DNA (DNA PCR-RAPD)	(Brandolini *et al.*, 2006)
Salt	Premium origin	Near-infrared (NIR) spectroscopy	(Galvis-Sánchez *et al.*, 2011)
Shellfish	Geographic origin	Inductively coupled plasma atomic emission spectroscopy	(Li *et al.*, 2017)
Vegetables	Organic production	Isotope ratio mass spectrometry	(Bateman *et al.*, 2007)
Vinegar	Wine vinegar authenticity	Isotope ratio mass spectrometry	(Camin *et al.*, 2013)
Fish	Fresh versus frozen-thawed discrimination	Front-face fluorescence spectroscopy	(Karoui *et al*., 2017)
Meat	Species adulteration	Visible and near-infrared (Vis and NIR)	(Rady and Adedeji, 2018)
Coffee	Adulteration	Diffuse reflectance Fourier-Transform Infrared (FTIR) spectroscopy	(Reis *et al*., 2013)
Veal	Adulteration with pork	Near-infrared (NIR) spectroscopy	(Schmutzler *et al.*, 2015)
Beef	Adulteration with pork	Near-infrared (NIR) spectroscopy	(Kuswandi *et al.*, 2015).
Honey	Adulteration with rice or corn	Fourier-Transform Infrared spectroscopy	(Limm *et al*., 2023)
Fish	Species authenticity	Raman spectroscopy	(Hu *et al*., 2023)
Fish	Geographical origin	Inductively coupled plasma mass spectrometry (ICP-MS)	(Correia *et al.*, 2012)
Crustaceans	Geographical origin	Inductively coupled plasma mass spectrometry (ICP-MS)	(Albuquerque *et al.*, 2016)

Matrix	Problem/incident	Analytical method	Reference
Crustaceans	Geographical origin	Inductively coupled plasma mass spectrometry (ICP-MS)	(Miller *et al.*, 2013)
Molluscs	Geographical origin	Inductively coupled plasma mass spectrometry (ICP-MS)	(Bennion *et al.*, 2019)
Molluscs	Geographical origin	Inductively coupled plasma mass spectrometry (ICP-MS)	(Zhao and Zhang, 2016)
Molluscs	Geographical origin	Inductively coupled plasma mass spectrometry (ICP-MS)	(Bennion *et al.*, 2021)
Molluscs	Geographical origin	Inductively coupled plasma mass spectrometry (ICP-MS)	(Ricardo *et al.*, 2015)
Molluscs	Geographical origin	Inductively coupled plasma mass spectrometry (ICP-MS)	(Morrison *et al.*, 2019)
Cereal products	Authenticity	Inductively coupled plasma mass spectrometry (ICP-MS)	(Pérez-Rodríguez *et al.,* 2023)
Honey	Botanical/geographical origin	Energy-dispersive X-ray fluorescence spectroscopy (ED-XRF)	(Ghidotti *et al.,* 2021)

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Dr Agnieszka Rupnik, FSAI

Secretariat to the Subcommittee

Ms Judith Giles, FSAI

Food Safety
AUTHORITY OF IRELAND

Food Safety Authority of Ireland
The Exchange, George's Dock, IFSC,
Dublin 1, D01 P2V6

T +353 1 817 1300
E info@fsai.ie

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