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To cite this article: Carolina González-Ferrero & María-José Sáiz-Abajo (2015) Characterization and stability studies of bioactive compounds and food matrices as evidence in support of health claims, International Journal of Food Sciences and Nutrition, 66:sup1, S4-S12

To link to this article: <http://dx.doi.org/10.3109/09637486.2015.1042843>



Published online: 04 Aug 2015.



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COMPREHENSIVE REVIEW

Characterization and stability studies of bioactive compounds and food matrices as evidence in support of health claims

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Abstract

The characterization and stability evaluation of food and food constituents (chemical active ingredient/microorganism) for which nutrition or health claims want to be requested are essential for the success of an application to EFSA. This work reviews the requirements that must be fulfilled for a full characterization of the active substance, comprising origin, elaboration, or extraction method, and chemical/microbiological composition, using validated analytical methods. The review focuses not only on establishing the specifications of the final active ingredient or food but also on ensuring homogeneity between batches. In addition, the article discusses the methodologies and conditions of the stability studies that need to be performed on food and food constituents to verify that the relevant compounds – chemical and microbiological active ingredients – will get to the consumer in the intended state and concentration to accomplish the claimed health effect over shelf life.

Keywords

Analytical methods, characterization, health claim, quality assurance, quality control, stability, validation

History

Received 5 December 2014

Revised 5 February 2015

Accepted 16 April 2015

Published online 31 July 2015

Introduction

In order to provide scientific evidence of the role an active ingredient plays on the consumer's health (food constituent), it is necessary to address a complete characterization of the bioactive compounds (BACs) which constitute the ingredient, and the BACs within the food matrix in which they are incorporated. This characterization needs to properly establish criteria for the standardization of the ingredients, their bioavailability, dose–response relationships, or the mechanistic principles governing their health effect.

This specificity in the identification process of the active ingredients is, in fact, required by EFSA according to the EU regulatory framework (Regulation (EC) No. 1924/2006). Multiple ingredients have been rejected due to the lack of a proper description (EFSA J, 9: 2243, 2011b). On several occasions, EFSA has concluded that the constituents of the food object of the review have not been properly characterized and, therefore, a causal relationship between these constituent and the claimed effect could not be established.

According the “Scientific and technical guidance for the preparation and presentation of an application for authorization of a health claim (revision 1) (EFSA J, 9:2170, 2011a)”, any new application must contain information about the characteristics of the *food/food constituent* for which a health claim is made.

This chapter tries to give some keys to be considered when completing Part 2 “Food/Food Constituent and its characteristics” of an application, which includes information relating to source and specifications, variability from batch to batch,

soundness and standardization, analytical methods, manufacturing process, stability information, and bioavailability data (developed in another article of this supplement, *Nutrikinetic studies of food bioactive compounds: from in vitro to in vivo approaches*).

This information is broken down into three parts:

- Food constituent
- Food or food category
- References

Characterization of food constituent and food matrix

Any food/food constituent (e.g. a nutrient or any other substance, or a combination of nutrients/other substances) for which the health claim is made should be characterized.

Name and characteristics

Source of the food constituent

The source and specifications (e.g. physical and chemical properties, composition and, where applicable, microbiological profile) of the food constituent for which the health claim is made should be provided.

The *source* of the food constituent should be described according to: plant taxonomy (genus identification), origin, typical edaphological conditions, part of the plant used to extract the active constituent or extract (leaves, root, whole plant, etc.), seasonality or harvesting time, typical concentrations found in plants.

Other characteristics are also welcome, such as conditions of transport of raw materials before extraction/processing or storage conditions.

When food constituents or BACs come from natural extracts, the species from which such compounds and/or extracts come from should be defined. For this purpose, it is possible to use botanical taxonomy that deals with the classification of plants similar to systematic botany.

Identification of plant species can be carried out through two tools:

- The field guides.
- Identification keys (dichotomous key)

Field guides are books in which a selection of the most significant species in a biome, region or area is presented. Some of them cover all species while others are specialized and dedicated to a single group of animals or plants (birds, flowering plants, etc.). Field guides are an important sector of the publishing market so the existing catalogue of titles is quite extensive. However, not all the world's regions are covered in field guides so in some cases it is necessary to settle for very general works.

An identification key is a method by which a plant is identified through the choice of contrasting or conflicting sets of characters. The most widespread identification keys are dichotomous keys in which two incompatible character sets are presented. The difficulty of handling these keys depends on the number of taxons covered by them. In general, the use of keys requires a basic knowledge of botany and sometimes it is difficult to use keys unless they are accompanied by a glossary. Once the key leads to identifying the species, the correctness of the identification must be verified through a field guide.

During the last few years, the use of molecular genetic methods for the conservation and the use of plant genetic resources have increased. International Plant Genetic Resources Institute (IPGRI) recognizes the considerable potential of molecular methods to improve the conservation and the use of plant genetic resources, and it now intends to consolidate the work in this area (Ayad, 1997).

The birth of molecular markers helped to eliminate the drawbacks both of a selection based on the unique phenotype analysis and identification of species and varieties in a more rigorous and reproducible way (Claros & Gonzalo, 2001). Advances in recombinant DNA technology allowed the development of molecular markers based on DNA, achieving stability in the identification of species and varieties (Claros & Gonzalo, 2001).

It is possible to estimate genetic diversity with molecular markers. There are different types of techniques that provide different types of information according to the characteristics of the molecule or molecule fragment analyzed. The most common approach is to detect differences in size from the frequencies listed of the alleles.

Random amplified microsatellites (RAMS) are small and repeated regions of DNA sequences, usually containing from two to three nucleotides, which may or may not be associated with genes. These regions are highly variable and often very useful for measuring similarities between species or related strains.

Zietkiewicz et al. (1994) developed a novel method for measuring the genetic diversity in plants and animals using a technique that combines the use of primers containing microsatellite sequences and a 5'-degenerated end. This technique combines the benefits of the microsatellites with the universal RAPD analysis (random amplification of polymorphic DNA). Hantula et al. (1996) proposed to name this technique as RAMS. They chose four primers (GT ACA CCA CGA), designed with a length of 18 bases including the 5'-degenerated end, which serves as an anchor to secure the attachment of the primer at the start of the microsatellite.

RAMS is based on polymerase chain reaction (PCR). It is a highly reproducible method and allows the detection of intra- and interspecific DNA polymorphisms. Patterns obtained with RAMS can be used for population studies (Bonilla & Espinosa, 2003). Another advantage of this technique is that it does not require the accurate estimation of the amount of DNA before the reaction, which is useful when many samples are analyzed within a

population, and also for the identification of species or strains (Bonilla & Espinosa, 2003; Cardozo & Silva, 2005).

Physico-chemical properties

The *physico-chemical properties* must be determined in the food constituent, this being either an extract or an individual compound. Solid/liquid state, water activity and water content, and physical aspect (dust, color, etc.) must be well established. In the case of dust materials, the particle size or range of particle size must be included in the specifications.

Composition

Composition of the food constituent is one of the most important characteristics to be determined. Especially, when working with a mixture of compounds (extract) in which only some of them are active. The whole characterization of the extract must be provided including that of each compound or component up to 99.9% of the weight of the sample. If the food constituent has undergone any stabilization process (e.g. microencapsulation), then the cover material used must be also considered in the composition of the food constituent.

If the subject of the claim is a food, then the category of food for which the health claim is made should be described. Moreover, a brief description of the food or food category, including characterization of the food matrix and the overall composition (including the nutrient content of the food), should be provided.

Food characterization should include not only the amount of active food constituent or BAC but also the macronutrients and major components such as:

- Humidity/Dry extract
- Ash content
- Protein
- Fiber
- Fat
- Carbohydrates

The source and specifications of the food or food category for which the health claim is made should be provided, and in particular the content of the constituent(s) related to the health claim.

For the identification and the characterization of the BACs, advanced analytical techniques are required. This includes identifying new substances, characterizing its structure and activity, as well as the critical factors of the quantification in the natural matrix and controlling the specifications of the product (Bernal et al., 2011).

Regarding analytical methods, it is necessary to use detectors able to not only identify compounds but also quantify them by retention time, wavelength, etc. Currently, gas or liquid chromatography tandem mass spectrometry is one of the analytical methods showing greatest potential for undertaking these aspects.

Validation of analytical methods is also a relevant issue. The methods developed should be validated in terms of precision, accuracy, and reliability (Betz et al., 2011).

The role played by high-performance liquid chromatography (HPLC) for the analysis of natural products is invaluable, considering that about 80% of the compounds of the sample are non-volatile and heat-sensitive (and, therefore, incompatible with gas chromatography) (Yang et al., 2009). The introduction of UPLC (ultra-performance liquid chromatography) methodologies has provided a performance improvement of the technique, especially concerning the sensitivity, speed, and resolution in the analytical methods (Zhao et al., 2011).

During the last decade, the use of liquid chromatography coupled to mass spectrometry has undergone tremendous

progress, both in terms of technology development and in terms of application in fields such as (bio) food security analysis, natural products (Malik et al., 2010), medicinal plants (Xing et al., 2007), and nutraceuticals (Gray et al., 2010). This technique is a powerful tool for characterization. It provides higher sensitivity (only a few micrograms of sample are needed) and, thanks to the high-resolution capacity of modern mass spectrometers, allows obtaining an extraordinary selectivity. Furthermore, this technique has the potential of providing information about the molecular weight and on the structure of analytes, using the features of MS-MS or MSⁿ (Marston, 2007).

In order to establish this methodology as a reference in structural analysis, many problems of compatibility between chromatographic systems and mass spectrometers had to be solved. The development of the interfaces such as electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI) provided a solution to analyze a wide range of molecules, from small non-polar to large polar molecules.

Among the different types of mass spectrometer, the ion trap has the unique ability to produce data that are important to MSⁿ structural elucidation, the time of flight detectors (TOF and qTOF) has the ability to provide maximum spectral resolution of the exact mass, and the triple quadrupole detectors MS-MS are the most appropriate bioanalytical assays that can also be used for metabolite identification.

In situations in which mass spectrometry is insufficient to establish the final structure of the molecules, NMR methodologies represent a complementary technique. Two-dimensional (2D) NMR techniques can be used for structural analysis without reference substances; however, without previous information about the molecular mass, these are complicated and complex methodologies due to the limited sensitivity and the need to isolate relatively large quantities of compound for analysis.

Other separation methods that have gained importance for the analysis of natural compounds are based on capillary electrophoresis, with enormous potential due to their different selectivity provided compared with HPLC (Gotti, 2009). However, their application is still limited and complex. No interfaces able to work with mass detectors are available due to incompatibilities in voltage (Valls et al., 2009), which makes difficult to obtain structural information out from the compounds separated by electrophoresis.

The characterization of microorganisms at levels of species and strain implies the use of molecular techniques such as denaturing high-performance liquid chromatography (DHPLC). This methodology can be used for characterization of probiotic strains. The DHPLC technique provides additional information in the genetic characterization of a strain of interest. In the case of microorganisms (e.g. bacteria and yeast), in addition to the identification of species, there must be a sufficient characterization (genotyping) at strain level using internationally accepted molecular methods. EFSA recommends that, during the manufacturing process, sufficient information is obtained to demonstrate the consistency of the final product. Besides, the strain characterization is essential so it can be differentiated from the rest of the gut microbiota in animal testing or intervention (Hafez & Elbestawy, 2009; Klein, 2010, 2011; Sarmiento Rubiano et al., 2010). In addition to DHPLC, other molecular techniques provide complementary information: sequencing all or part of the genome of the strain of interest, RAPD and RFLP (restriction fragment length polymorphism), which allow differentiation at strain level.

The fundamentals of the DHPLC technique are described next. The amplification of the conservative fragments appropriate for strain differentiation (e.g. 16S and *ldh*) is performed starting from the chromosomal DNA of the different probiotic strains. For this purpose, specific design of primers and optimization of PCR

amplification are required. Once the amplicons are obtained, their identification and separation is performed by DHPLC. Different methods for identification of strains based on differences in the regions studied still need to be developed.

The strategy of choice can be used to amplify each strain separately. However, multiplex PCR with DNA mixture of different strains could also be performed in order to evaluate the discrimination ability of the DHPLC by splitting and sequencing the different peaks obtained.

In this regard, the DHPLC technique allows differentiating subpopulations present in a mixture based on differences in gene sequence. This technique offers several advantages over traditional methods of analysis of microbial communities in gels (DGGE, denaturing gradient gel electrophoresis and terminal T-RFLP) allowing discrimination at the species level. The main advantages are higher sensitivity and effectiveness, reduction in time of analysis, and reduction in methodological difficulty of the technique (gel preparation, electrophoresis, detection of bands using carcinogenic reagents, and band re-amplification for sequencing). Besides, the process is highly automated, allowing us to work with a large number of samples, even semi-quantitatively compared with the use of other techniques such as DGGE. It also allows collecting peaks to work with them afterwards by improving the difficulties of retrieving the bands from gels.

The DHPLC technique has been used since the 1990s in the field of biomedical research in order to detect mutations and polymorphisms in DNA (Narayanawami & Taylor, 2001; Wagner et al., 1999). The use of this technique to identify microbial populations has recently emerged (Barlaan et al., 2005; Hurtle et al., 2002) as a new application of this technology.

With the ability of DHPLC to identify sequence variations, it is possible to identify and monitor microbial populations in different environments such as the gastrointestinal tract (Goldenberg et al., 2005, 2007), buccal and gingival flora (Jacinto et al., 2007), or seawater (Barlaan et al., 2005).

DHPLC is a technique able to study dynamics of population and biodiversity without the need to first isolate the microorganisms in a culture medium. Thus it allows, for example, tracking the presence of the strains of interest in faeces, in the intestine, as much as its viability, proportions, etc. in order to associate a profile of peaks with certain health conditions, antibiotic treatments, or consumption of probiotics (metagenomics). In fact, DHPLC has already been successfully used to monitor the intestinal flora of hospitalized patients in order to assess the effect of a treatment with antibiotics on the same (Goldenberg, 2005). The DHPLC technique has been compared with other molecular techniques for characterizing and monitoring the development of the intestinal flora of infants, being the most advantageous in comparison with the other techniques (Klein, 2011).

Contaminants

Finally, a detection of *contaminants* present in the food constituent, extract, or food should be provided. This characterization should include the following:

- Microbiological contaminants (human pathogens and others).
- Residual solvents (from the extraction method used).
- Residues: pesticides, heavy metals, mycotoxins, PAHs (polycyclic aromatic hydrocarbons).
- Other residues (if applicable): acrylamide.

In particular, when food constituents have been obtained through an extraction process using organic solvents, it is important to measure the solvent residues that are present in the food ingredient after the manufacturing process. With the Royal

Decree 1101/2011 of 22 July published in BOE on 30 August 2011 the Spanish legal system incorporates Directives 2009/32/EC and 2010/59/UE and expressly repeals Royal Decree 472/1990 of 6 April. In this legal document, the extraction solvents used in the manufacture of food products and their ingredients are regulated. The main objective of these regulations is to approve the positive list of extraction solvents that are allowed for use in the manufacture of food products and ingredients, which are set out in the Annex to that regulation.

In the Article 5, the criteria to be fulfilled by such solvents are included:

- They must not contain a toxicologically dangerous quantity of any reagent or substance.
- Lead and arsenic content must be less than 1 mg/kg.
- They must respond to specific purity criteria set out in the Annex.

Besides, the Royal Decree regulates the information that must appear on the labeling of these solvent products used for extraction.

Variability from batch to batch

The *variability from batch to batch* should be addressed and documented. Different productions in conditions of maximum variability scenarios should be performed and the quantification-characterization of the food/food constituent should be addressed.

Conditions of maximum variability include each factor that may affect the content or quality of the BAC: different geographical locations of the plants, different seasons and parts of the plant used, admitted tolerance in production/extraction process parameters (times, temperatures, etc.), differences in solvent or equipment used in the production, personnel, etc.

The variability in the food constituent or food obtained in each of these production batches performed at maximum variability scenarios can be considered as values for actual inter-batch variability.

Analytical methods applied

The *analytical methods applied* should be scientifically sound and standardized to ensure the quality and consistency of the data. These analytical methods should follow the guidelines described for the characterization of the BAC of food constituent above mentioned.

The analytical methods used should be validated in the analytical laboratory to be implemented. Adjustment and refinement to specific laboratory conditions is required as well as the quality assurance (QA) and quality control (QC) procedures necessary to improve reliability of the results when applying an established analytical method. These are important practical requirements necessary to trustfully apply an analytical method in a laboratory.

The analytical methods used in the characterization of the BACs, food constituents, and nutrient analysis of foods and macronutrients should be validated according to appropriate guidelines (UNE-EN ISO/IEC 17025:2005; Eurachem Guide and ENAC Guides for method validation).

In order to demonstrate that a method is valid for the intended use, a number of parameters to be determined are proposed, depending on the type of method used:

- Accuracy
- Accuracy (repeatability, reproducibility)
- Selectivity/specificity
- Working range
- Linearity/response function
- Limits of detection and quantification
- Uncertainty

Table 1. Type of analysis and parameters to be considered in each case for the characterization and the stability studies of food and food constituents.

Type of analysis	Parameter
Identification	Specificity/selectivity Uncertainty
Quantitative analysis/stability studies	Working range Response function/linearity Selectivity/specificity Precision Accuracy Quantification limit Uncertainty
Qualitative analysis	Specificity/selectivity Detection limit

The values that must meet these parameters depend on the intended use of the analytical method. In those methods in which the measurement is performed directly, the equipment must be properly calibrated and verified.

In the case of direct physical methods, validation must take into account the reproducibility of the technique (if this is possible), and, in some cases, the accuracy should be established if reference materials are available. In the case of destructive physical analysis, a reproducibility study on the same sample is not possible, as the test sample on which the test has to be performed cannot be repeated. Table 1 defines general criteria to select which parameters should be determined depending on the type of analysis performed.

The quantification of the food constituent in the food matrix should be included and reported by the analytical methods and quality assurance described before. Besides, the matrix effect should be assessed to prove that the analytical methods used are able to quantify the BACs in the specific food matrix.

Measurements should be performed in a competent laboratory able to certify the data obtained. Whenever a quality system is in place for control/documentation (e.g. good laboratory practices, GLP, and ISO17025), this particular system should be indicated.

Manufacturing process

Where applicable, a brief overview should be provided, and if a quality system is followed in the production process (e.g. good manufacturing practices, GMP), this particular system should be indicated.

The description of the process applicable for food constituents should include the following:

- Type of extraction process used: fundamentals.
- Relation of each of the components used in the extraction process. Solid/solvent ratios, adjuvants, reagents used, etc.
- Process conditions (times, temperatures, pressures).
- Scale of the process (kg per batch).
- Quality system: GMP applied
- Yields obtained

Regarding the manufacturing process for the specific food, the description of the process should include the following:

- General formulation process including the BAC for which the health claim is formulated
- Process conditions (time, temperature, pressure, etc.)
- Scale of the process (kg per batch)
- Quality system: GMP applied
- Quality controls applied on the final produce

The most common extraction methods to obtain phytochemicals, extracts, and essential oils are the following:

- **Hydrodistillation:** Distillation is the separation of the components of a sample based on their different vapor pressures and boiling points. A mixture of components does not have a specific boiling point (a temperature) but a range of temperatures that depend on the components of the mixture. Different oils have different boiling points and the distillation process occurs usually between 150 and 300 °C. As a result of the distillation, the components of a mixture are separated into two immiscible phases it is called hydrodistillation.
- **Supercritical fluid extraction:** Supercritical fluid extraction comprises the use of solvents in the supercritical point (pressure and temperature combination) that have a great solvent power and a huge ability to penetrate solids, allowing the rapid and almost complete depletion of extractable solids. Thus, solids can be fully and easily separated from the extracts only by changing pressure or temperature and, if necessary, the fluid can be passed into the gaseous state.

Occasionally co-solvents may be used to promote extraction (e.g. ethanol or water). The main disadvantage of supercritical fluids is the extraction time, which is usually long.

- **Solvent extraction (solid–solid, liquid–liquid):** Solvent extraction is carried out by extraction with organic solvents, which penetrate the plant matter and dissolve inner substances, which are evaporated and concentrated at low temperature. Then, the solvent is removed, obtaining the desired fraction.

Solvent extraction requires a larger investment than steam distillation, but generates higher yields compared to previous methods, and “almost all” the compounds present in the herb matrix can be extracted: volatile, fats, waxes, pigments, etc. Vacuum equipment is needed to obtain absolute oils and separate the solvent from the oils. It is also necessary to establish additional purification steps if the substance or product is produced for human consumption or for hygiene purposes.

- **Centrifugation extraction:** The extracts and oils obtained by this process are of higher quality in terms of aroma characteristics than those achieved by steam stripping. Centrifugation is not a thermal process and produces high-quality extracts. However, the internal friction of the raw material causes uncontrollable increase in temperature that may involve thermal degradation and browning of the extract. This problem requires the use of additional purification equipment with high operating costs that increase the final price of the product.
- **Solid phase extraction:** Cartridges and preparative columns are used to retain the analyte that is afterwards eluted with a small solvent volume.

Stability studies

If the application for which the health claim is made is related to a food constituent (e.g. the nutrient or other substance, or a combination of nutrients/other substances), the food constituent should be characterized as aforementioned. In both cases, stability information for both food and constituents should be provided. Where applicable, a summary of the studies undertaken (e.g. conditions, batches, and analytical procedures), and of the results and conclusions of the stability studies, should be disclosed. The conclusions concerning storage conditions and shelf life should be also provided.

According to EFSA guidance (EFSA J, 9: 2170, 2011a), the measurements should be performed in a competent laboratory where the data can be certified and the quality system established in the laboratory is indicated.

As specified in the Regulation, the quantity of the food/food constituent and pattern of consumption required to obtain the

claimed effect could reasonably be achieved as part of a balanced diet. Therefore, in order to ensure that the claims made are truthful, it is necessary that the substance that is the food/food constituent object of the claim is present in the final product in quantities that are sufficient to produce the nutritional or physiological effect claimed. The substance should also be bioavailable. These aspects will be considered in a different article within this supplement, *Nutrikinetic studies of food bioactive compounds: from in vitro to in vivo approaches*.

In addition, and where appropriate, a significant amount of the substance producing the claimed nutritional or physiological effect should be provided by the quantity of the food that can reasonably be expected to be consumed (Regulation (EC) No. 1924/2006).

Factors influencing stability

Many factors can influence the shelf life of food/constituent, which can be classified into intrinsic and extrinsic factors (IFST, 1993).

- **Intrinsic factors** are the properties of the final food/constituent:
 - Water activity, a_w (available water)
 - pH value, total acidity, and type of acid
 - Redox potential (E_h)
 - Available oxygen
 - Nutrients content and availability
 - Natural microflora and surviving microbiological counts
 - Natural biochemistry of the food/food constituent formulation (enzymes, chemical reactants)
 - Use of preservatives
 - Formulation/composition of food/constituent
- **Extrinsic factors** are those factors that can be modified during the processing, storage and/or distribution and influence of the final food/food constituent on shelf life:
 - Time-temperature profile during processing/other parameters from novel preservation technologies (high hydrostatic pressure, HHP; radiofrequency, RF; pulsed electric field, PEF; etc.)
 - Pressure in the headspace
 - Temperature control during storage and distribution
 - Hazard analysis and critical control points (HACCP)
 - Relative humidity (RH)
 - Exposure to UV and IR radiation
 - Environmental microflora counts
 - Composition of atmosphere within packaging
 - Subsequent heat treatment (e.g. reheating or cooking before consumption)
 - Consumer handling

Both the external conditions of the food and the intrinsic parameters can lead to biochemical and microbiological deteriorations.

Types of deterioration

The above-mentioned factors can interact by stimulating different deteriorative processes which limit the stability of a product (Adegoke, 2004; Blackburn, 2000; Kilcast & Subramaniam, 2000). These processes can be classified as the following:

- (1) **Microbiological:** The growth of certain bacteria, yeasts, and moulds in food/food constituent may lead to either food spoilage or food poisoning. The time necessary for microorganisms to affect food/food constituent will depend on some parameters such as initial microbial loading, moisture content, pH, presence of preservatives, processing method, storage conditions (time, temperature, and relative humidity), etc.
- (2) **Chemical:** Rancidity, enzymatic reactions, non-enzymatic browning, warming – over flavor, textural changes, etc. can

produce undesirable flavors and loss of nutrients or bioactivity.

- (3) *Physical*: Moisture migration, fat migration, permeability changes and subsequently, modifications in packaging atmosphere, etc.
- (4) *Temperature related*: For instance: changes in crystallization processes, destabilization of emulsion systems, etc. Moreover, temperature changes can increase or decrease the speed of other forms of spoilage.

Other factors such as those derived from storing food/food constituent near other strongly smelling products can also influence in food/food constituent stability.

Therefore, the combination of different deteriorative processes can lead not only the end of the shelf life of the product but also the production of inactive biologically and/or even toxic substances.

Consequently, it is necessary to carry out the stability studies of the food/food constituent to set specifications that ensure their quality and safety as well as the health claimed property.

How to measure the stability of food/constituent

In order to determine the stability of the food/constituent, the following should be considered.

Sensory evaluation

Assessment of sensory shelf life can be approached in one or two ways: from the measurement of sensory characteristics or from a consumer preference approach. In the perception of food quality, all senses play an important role in human beings: vision, gestation, olfaction, chemical/trigeminal, touch, and hearing. This perception derives in the quality parameters: appearance, taste, odor/aroma, irritation, and texture. The combination of taste, odor/aroma, and the perception of irritation is named flavor.

The complex nature of food quality perception and the differences among human subjects to perceive creates difficulties to use sensory evaluation as a reliable instrument to measure the stability of a product. In order to ensure the accuracy, precision and validity of these analyses, it is essential to select and train subjects suitable for performing sensory evaluation.

There are two types of sensory test procedures:

- Analytical tests are used to determine the differences in terms of nature and magnitude, and thereby to evaluate the stability of food/constituent. For this purpose, a panel consisting of 6–10 (Foegeding et al., 2011), 6–12 (Drake, 2007), or 8–16 (Brookfield et al., 2011) members who have been trained in sensory evaluation methodologies (Chen & Opara, 2013) is required.
- Hedonic/preference or purchase intentions tests are used to determine the preference of consumer liking. In this case, the sensory evaluation panel is recommended to be composed of untrained product consumers, and the number of panelists is expected to be higher and at least 70 (Civille & Oftedal, 2012).

Sensory experiments can generate large amounts of data, and their validation by using statistical techniques is required to establish reliable conclusions.

If valid instrumental measurement methods are available, e.g. rheological analysis to evaluate texture, these can be useful to supplement the sensory data. Their value can be mostly found in long-term measurement of shelf life which poses substantial challenges to the sensory analyst.

Physical analysis

The main physical test measures the changes in texture (Lan & Umezurike, 2013). The instrumental measurement of food

texture allows overcoming the limitations of sensory evaluation affected by adaptation and fatigue (Peleg, 2006), and level of training received by the panellists (Bárcenas et al., 2007).

The rheological changes can be induced by chemical reactions produced by interaction of constituents or by external factors. The method of measurement of texture has to be chosen carefully depending on the product in order to obtain a correlation between instrumental analysis and sensory tests. Examination of packaging and “travel tests” provides additional information to determine the best, worst, and average retail conditions.

A “travel test” helps to identify any hazards involved in transport and handling through the expected distribution and storage chain. An examination of the product at various points and at the end of the chain is required (Booklet for the Food Industry, 2005). Experimental design should attempt to mimic real life practices (e.g. include predicted transport temperatures to retail outlets, during commercial control, consumer purchase and transport, and consumer storage) (Kilcast & Subramaniam, 2000).

Chemical analysis

Many chemical reactions can occur in a product during storage, which can influence the stability of the food/food constituent and consequently the bioactivity of its components. In order to ensure that the dose and the nature of food/food constituent have not suffered any changes during storage, it is necessary to evaluate these components using scientifically standardized analytical methods. For example, if the health claim is related to the dose of a number of polyphenols from a vegetable or a fruit, the concentration and the stability of these compounds must be demonstrated by validated chemical analysis throughout the shelf-life of the food/food constituent (EFSA J, 11: 3084, 2013a).

Microbiological analysis

Microbiological analyses should be focused on three aspects:

- Microbial growth, which causes the spoilage of the food/constituent.
- The growth of microbial pathogens compromising the safety of the food/food constituent.
- If the health claim is related to the presence of a specific/or blend of microorganisms, then the characterization by molecular strain typing should be provided. Moreover, the information related to the stability of such microorganisms in the food/food constituent and its microbiological safety should be specified (EFSA J, 11: 3086, 2013b).

The “time to spoilage” can be calculated by measuring the microbial load at staged intervals during storage of the food/food constituent at the appropriate temperature. The time to reach a pre-determined level of total microbial count and a pre-determined level of individual microbes can be used to determine the end-point. According to Patrick (2000), it is advisable to leave a safety margin in setting the shelf life. Generally, this margin is 70% of time to spoilage.

Currently, mathematical modeling can be used to identify specific organisms that may cause food safety problems and to predict their rate of growth in food/constituents.

Design of stability/shelf-life tests

Shelf life is usually defined as the time during which a food product remains safe, complies with label declaration of nutritional data, and retains its desired sensory, chemical, physical, and microbiological characteristics when stored under the recommended conditions (Giménez et al., 2012; IFST, 1993).

Therefore, to assess shelf-life objective indexes related to nutrition, microbiological or physicochemical characteristics of food have been typically measured (Cardello, 1995; Wansink & Wright, 2006).

During its shelf life, a food/food constituent should:

- Remain safe to eat.
- Keep its hedonic/quality parameters.
- Meet both the nutritional and health claims provided on the label.

In the particular case of products bearing health claims, the constituent/s responsible for that health claim must be monitored. Shelf-life tests are a series of experiments designed to obtain information on the stability of a food/food constituent in order to define its shelf life under certain conditions of processing, packaging, and storage (temperature and relative humidity).

In order to characterize the stability of a particular food/constituent, it is necessary to study its decomposition or degradability under controlled conditions of temperature, humidity, and light, in the packaging where it is to be marketed. The stability also depends on its formulation and processing, type of container, and closure system selected by the manufacturer. Therefore, in the stability studies performed for the technological development of the product is necessary to take into account possible interactions between the different ingredients and their interactions with the packaging material in which they will be distributed, transported, and stored along its shelf life. Shelf life is determined for the climatic zone where the product is marketed. The recommended storage conditions established during stability studies for a given product should ensure quality, safety, and the efficacy of the effects attributed to the product over its shelf life. Moreover, adverse weather conditions should be considered in certain countries where the food/food constituent may be exported.

The results of the stability studies are used to determine the shelf life and the stability of food/food constituent while maintaining its healthy properties, thereby confirming its intended shelf life, and recommended storage conditions.

The two main methods to determine the shelf life of food/food constituent are the following:

Direct method: Designed by Gacula & Kubala (1975) and also called a partially staggered design. It involves storing the product (a single batch or replicate batches) under controlled conditions similar to those in which the food/food constituent will be kept during storage once it has been launched into the market, for a period longer of time than the expected shelf life. During this period, samples are taken off for testing at intervals determined by the expectation of the probable shelf life. If there is no prior knowledge about this, it may be necessary to take sufficient samples at each time point.

Indirect method: This methodology is usually performed in different situations: prior to the launch of new healthy food products, for compliance with labeling requirements, or for the products with a long shelf-life. These procedures aim to predict shelf-life without carrying out a full length storage trial.

The two most common indirect methods are the following:

- Accelerated shelf-life studies

The trial period is shortened by deliberately increasing the rate of deterioration. This is usually done by increasing the storage temperature. The results are then used to estimate the product's shelf life under normal storage conditions. Such methods can only be used if there is a known and validated relationship between the storage characteristics under an ambient storage and the storage characteristics under an accelerated condition.

The basic premise of an accelerated test is that by changing a storage condition, the chemical or physical process that leads to

spoilage are accelerated, and that a predictive shelf-life relationship related to ambient conditions can be defined. That is, that the deteriorative process limiting shelf life remains the same under the two conditions. This relationship is not attainable if the deteriorative process at abuse conditions is different with regard to the ambient conditions.

It is remarkable that accelerated conditions cannot be achieved by only raising the storage temperature using an Arrhenius model since this model is only appropriate for simple chemical systems (Blackburn, 2000). R programming and advanced non-linear models are usually employed to model accelerated conditions in more complex chemical systems.

These studies are not always predictive of physical changes. Therefore, it is necessary that the accelerated method is validated. Thus, the stability of the food/food constituent must be tested not only at recommended storage conditions by manufacturer but also at abuse conditions that may be encountered through the distribution chain and in the consumer environment.

- Predictive modeling

Predictive models are mathematical equations which use information from databases to predict bacterial growth under defined conditions. Most of them are specific to particular types or microorganisms (e.g. Baseline 1.0, <http://www.baselineapp.com>). Such models look for statistical and mathematical relationship between three sets of variables: intrinsic (product related) factors, extrinsic (environment) factors; and implicit factors (characteristics of the microorganism itself and how it behaves in the presence of combinations of intrinsic and extrinsic factors). Such models need to be based on the good experimental data mapping rates of change within given combinations of factors.

The data from these experiments are analyzed for statistical patterns and mathematical relationships from which a model can be built.

It is important to consider that information from modeling programmes needs to be verified by challenge testing or by a shelf-life trial. Challenge testing is used to assess whether the formulation of a food product and its storage conditions can be used to control the growth of pathogens, if present, during the designated shelf life.

This procedure involves inoculation of a product with relevant microorganisms and its incubation under controlled environmental conditions in order to assess the risk of food poisoning, or to establish product stability. When the effect of such food control systems on specific pathogenic microorganisms cannot be predicted from the literature, it may be necessary to use challenge testing to evaluate the safety of the product.

Therefore, when a new product has been developed, accelerated stability tests can be performed whenever possible to compare different formulations, manufacturing processes, or packaging materials in a faster way. Once defined the final formulation or food component, processing conditions, and packaging of the final product to be marketed, *stability tests at normal conditions* should be carried out, in order to confirm the results of accelerated stability tests. Stability must be shown for both the food and food constituent in each formulation, processing conditions and packaging of the product for final consumption in order to establish shelf life. Whenever major changes are made in the formulation, manufacturing process or packaging additional stability studies are required, substantiating product quality, and the health claim submitted (concentration and bioavailability).

Conclusions

In conclusion, a complete characterization of food constituent or food should be provided for any new health application,

Box 1. Practical approaches.

- Complete analytical and/or microbiological characterization is required using appropriate and validated methods.
- Specifications must be established to ensure reproducibility between batches.
- Stability information must be provided to ensure an effective dose of bioactive compounds during the intake and along shelf-life of the food.

identifying and quantifying the active compounds responsible of the demonstrated bioactivity. The main aspects to be considered in relation to characterization and stability studies of BACs are summarized in Box 1. This characterization must include different aspects comprising raw materials such as provenance and harvesting period and quality parameters of these materials. In addition, processing methods must be established to guarantee the reproducibility between batches. Quantitation is necessary not only to establish a dose–response or cause–effect relationship during the evaluation of bioactivity but also to ensure a certain intake level by serving of food for the consumers. Therefore, analytical methods used for this purpose must be validated and performed by competent laboratories.

Additionally, stability studies must be provided at standard conditions for both the food and the food constituent. In order to determine stability specifications, final formulation, processing, packaging, storage conditions and processing conditions by consumers (e.g. cooking) must be taken into account to design the analysis. The stability of the different constituents is required to ensure that consumers will have an intake of the active compound in the chemical form and in the quantity needed to achieve the health effect claimed. It is remarkable that whenever accelerated studies were performed, the methods must also be validated.

Acknowledgements

The authors also wish to acknowledge Dr Ana Romo Hualde (Centre for Nutrition Research) for her great support and help all along the development of the project INCOMES (Guide for the Support of Health Claims in foods: Immune and Cognitive functions and Metabolic Syndrome).

Declaration of interest

The authors report that they have no conflicts of interest. This work has been supported by the Project INCOMES (Barry Callebaut-La Morella Nuts SA, Biosearch SA, Biotecnologías Aplicadas SA, Bodega Matarromera SL, Miguel Torres SA, Galletas Gullón SA, Iberfruta SA, Laboratorios Ordesa SL, Newbiotechnic SA and Soria Natural SA), co-funded by the Spanish Ministerio de Economía y Competitividad (Centro para el Desarrollo Tecnológico Industrial) and FEDER. Also CIBERObn and SEN (Spanish Society of Nutrition) are gratefully acknowledged for their overall support concerning the presentations of this guide.

References

Adegoke GO. 2004. Understanding food microbiology, 2nd ed. Ibadan, Nigeria: Alleluia Ventures.

Ayad WG, Hodgkin T, Jaradat A, Rao VR. 1997. Molecular genetic techniques for plant genetic resources. In: Ayad WG, Hodgkin T, Jaradat A, Rao VR, editors. Report of an IPGRI Workshop 9–11 October 1995. Rome, Italy: IPGRI. p 47.

Bárceñas P, Elortondo Fr JP, Albiu M, Mège J, Roseiro LB, Scintu MF. 2007. An international ring trial for the sensory evaluation of raw ewes' milk cheese texture. *Int Dairy J* 17:1139–1147.

Barlaan EA, Sugimori M, Furukawa S, Takeuchi K. 2005. Profiling and monitoring of microbial populations by denaturing high performance liquid chromatography. *J Microbiol Methods* 61:399–412.

Bernal J, Mendiola JA, Ibáñez E, Cifuentes A. 2011. Review: advanced analysis of nutraceuticals. *J Pharm Biomed Anal* 55:758–774.

Betz JM, Brown PN, Roman MC. 2011. Review: accuracy, precision and reliability of chemical measurements in natural products research. *Fitoterapia* 82:44–52.

Blackburn CW. 2000. Modelling shelf-life. In: Kilcast D, Subramaniam P, editors. The stability and shelf-life of food. Cambridge: CRC Press, Woodhead Publishing Limited. p 55–75.

Bonilla BML, Espinosa PK. 2003. Colección, Caracterización Fenotípica y Molecular de poblaciones de uchuva *Physalis peruviana* L. Trabajo de Grado. Ingeniero Agrónomo. Universidad Nacional de Colombia. Sede Palmira.

Booklet for the Food Industry. 2005. A guide to calculating the shelf life of foods. Published by New Zealand Food Safety Authority Published in February 2005. Wellington, New Zealand. ISBN 0-478-07865-X Available at: http://www.foodsafety.govt.nz/elibrary/industry/guide_-_calculating-contains_background.pdf. Accessed on 3 November 2014.

Brookfield PL, Nicoll S, Gunson FA, Harker FR, Wohlers M. 2011. Sensory evaluation by small postharvest teams and the relationship with instrumental measurements of apple texture. *Postharvest Biol Tec* 59:179–186.

Cardello AV. 1995. Food quality: relativity, context, and consumer expectations. *Food Qual Prefer* 6:163–170.

Cardozo BC, Silva AB. 2005. Análisis de la Diversidad Genética de *Ralstoniasolanacearum* agente causal de la enfermedad del Moko en plátano y banano, utilizando la técnica RAMS en aislamientos colombianos. Trabajo de Grado para Ingeniero Agrónomo. Universidad Nacional de Colombia Sede Palmira. 109 p.

Chen L, Opara UL. 2013. Texture measurement approaches in fresh and processed foods. A review. *Food Res Int* 51:823–835.

Civille GV, Oftedal KN. 2012. Sensory evaluation techniques – make “good for you” taste “good”. *Physiol Behav* 107:598–605.

Claros D, Gonzalo M. 2001. Marcadores Moleculares: Qué son, cómo se obtienen y para qué valen. Available at: <http://www.encuentros.uma.es/encuentros49/marcadores.html>. Accessed on 12 June 2014.

Drake MA. 2007. Invited review: sensory analysis of dairy foods. *J Dairy Sci* 90:4925–4937.

EFSA J, 11: 3084. 2013a. Scientific opinion on the substantiation of a health claim related to monacolin K in SYLVAN BIO red yeast rice and maintenance of normal blood LDL-cholesterol concentrations pursuant to Article 13(5) of Regulation (EC) No 1924/2006. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). EFSA J 11: 3084–3097.

EFSA J, 11: 3086. 2013b. Scientific opinion on the substantiation of a health claim related to a combination of *Bifidobacterium longum* LA 101, *Lactobacillus helveticus* LA 102, *Lactococcus lactis* LA 103 and *Streptococcus thermophilus* LA 104 and improves stool frequency pursuant to Article 13(5) of Regulation (EC) No 1924/2006. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). EFSA J 11: 3086–3095.

EFSA J, 9: 2170. 2011a. Scientific and technical guidance for the preparation and presentation of an application for authorisation of a health claim (revision 1). EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). EFSA J 9:2170–2206.

EFSA J, 9: 2243. 2011b. Scientific opinion on the substantiation of health claims related to: dairy products (ID 1140, 1141, 1191), raw or processed food products of animal origin, plus bread and panification products (ID 1193, 1194), herbal yeast plasmolysate (ID 1815, 1816), apple polyphenols (ID 2713), rye flour (ID 1266), tomato juice (ID 1202), whey protein and alphasalalbumin (ID 424, 430, 432, 725, 1433), “brocco shoots”, “broccoli sprout powder” and “Brassica oleracea var. italica (broccoli)” (ID 1362, 1481, 2844, 2845), honey (ID 1159, 1160, 1318, 4678, 4679), and Cucurbitapepo L. (pumpkin) seeds and seed extracts (ID 2029, 2365) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). EFSA J 9:2243–2276.

Foegeding EA, Daubert CR, Drake MA, Essick G, Trulsson M, Vinyard CJ. 2011. A comprehensive approach to understanding textural properties of semi and soft-solid foods. *J Texture Stud* 42:103–129.

Gacula MC, Kubala JJ. 1975. Statistical models for shelf life failures. *J Food Sci* 40:404–409.

Giménez A, Ares F, Ares G. 2012. Sensory shelf-life estimation: a review of current methodological approaches. *Food Res Int* 49:311–325.

- Goldenberg O, Herrmann S, Adam T, Marjoram G, Hong G, Göbel UB, Graf B. 2005. Use of denaturing high-performance liquid chromatography for rapid detection and identification of seven *Candida* species. *J Clin Microbiol* 43:5912–5915.
- Goldenberg O, Herrmann S, Marjoram G, Noyer-Weidner M, Hong G, Bereswill S, Göbel UB. 2007. Molecular monitoring of the intestinal flora by denaturing high performance liquid chromatography. *J Microbiol Methods* 68:94–105.
- Gotti R. 2009. Review: capillary electrophoresis of phytochemical substances in herbal drugs and medicinal plants. *J Pharm Biomed Anal* 55:775–801.
- Gray MJ, Chang D, Zhang Y, Liu J, Bensoussan A. 2010. Development of liquid chromatography/mass spectrometry methods for the quantitative analysis of herbal medicine in biological fluids: a review. *Biomed Chromatogr* 24:91–103.
- Hafez EE, Elbestawy E. 2009. Molecular characterization of soil microorganisms. Effect of industrial pollution on distribution and biodiversity. *World J Microbiol Biotechnol* 25:215–224.
- Hantula J, Dusabenyagasan IM, Hamelin RC. 1996. Random Amplified Microsatellites (RAMS) a novel method for characterizing genetic variation within fungi. *Eur J Path* 26:159–166.
- Hurtle W, Shoemaker D, Henschel E, Norwood D. 2002. Denaturing HPLC for identifying bacteria. *Biotechniques* 33:386–388, 390–391.
- IFTS: Institute of Food Science and Technology. 1993. Shelf life of foods-guideline for its determination and prediction. London: IFTS.
- Jacinto RC, Gomes BP, Desai M, Ranjendram D, Shah HN. 2007. Bacterial examination of endodontic infections by clonal analysis in concert with denaturing high-performance liquid chromatography. *Oral Microbiol Immunol* 22:403–411.
- Kilcast D, Subramaniam P, editors. 2000. Introduction. In: *The stability and shelf-life of food*. Cambridge: CRC Press, Woodhead Publishing Limited. p 1–19.
- Klein G. 2010. Antibiotic resistance and molecular characterization of probiotic *Lactobacillus* strains and clinical safety in relation to aspects of probiotics. *Foodborne Pathog Dis* 8:267–281.
- Klein G. 2011. Molecular characterization of the probiotic strain *Bacillus cereus* var. *toyoi* NCIMB 40112 and differentiation from food poisoning strains. *J Food Protect* 74:1189–1193.
- Lan C, Umezuruike LO. 2013. Texture measurement approaches in fresh and processed foods – a review. *Food Res Int* 51:823–835.
- Malik AK, Blasco C, Picó Y. 2010. Liquid chromatography-mass spectrometry in food safety. *J Chromatogr A* 1217:4018–4040.
- Marston A. 2007. Review: role of advances in chromatographic techniques in phytochemistry. *Phytochemistry* 68:2785–2797.
- Narayanaswami G, Taylor PD. 2001. Improved efficiency of mutation detection by denaturing high-performance liquid chromatography using modified primers and hybridization procedure. *Genet Test* 5:9–16.
- Patrick M. 2000. Leatherhead Food RA, personal communication.
- Peleg M. 2006. On fundamental issues in texture evaluation and texturization – a view. *Food Hydrocolloid* 20:405–414.
- Regulation (EC) No. 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. Available at: <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:012:0003:0018:EN:PDF>. Accessed on 30 October 2014.
- Sarmiento-Rubiano LA, Berger B, Moine D, Zúñiga M, Pérez-Martínez G, Yebra MJ. 2010. Characterization of a novel *Lactobacillus* species closely related to *Lactobacillus johnsonii* using a combination of molecular and comparative genomics methods. *BMC Genomics* 11:504.
- Valls J, Millán S, Martí MP, Borrás E, Arola L. 2009. Advanced separation methods of food anthocyanins, isoflavones and flavanols. *J Chromatogr A* 1216:7143–7172.
- Wagner T, Stoppa-Lyonnet D, Fleischmann E, Muhr D. 1999. Denaturing high-performance liquid chromatography BRCA1 and BRCA2 reliably detects mutations. *Genomics* 62:369–376.
- Wansink B, Wright AO. 2006. “Best if used by...” How freshness dating influences food acceptance? *J Food Sci* 71:354–357.
- Xing J, Xie C, Lou H. 2007. Recent applications of liquid chromatography-mass spectrometry in natural products bioanalysis. *J Pharm Biomed Anal* 44:368–378.
- Yang M, Sun JH, Lu ZQ, Chen GT, Guan SH, Liu X, Jiang BH, et al. 2009. Phytochemical analysis of traditional Chinese medicine using liquid chromatography coupled with mass spectrometry. *J Chromatogr A* 1216:2045–2062.
- Zhao J, Lv GP, Chen YW, Li SP. 2011. Review: advanced development in analysis of phytochemicals from medicine and food dual purposes plants used in China. *J Chromatogr A* 1218:7453–7475.
- Zietkiewicz E, Rafalski A, Labuda D. 1994. Genome fingerprinting by simple sequence repeat (SSR) anchored polymerase chain reaction amplification. *Genomics* 20:176–183.