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2. CHARACTERIZATION OF THE SPICES & HERBS PROPERTIES: METHODS AND TECHNIQUES

Spices and herbs are widely used as food flavoring, seasoning, and coloring agents and sometimes as preservatives. Not only are spices used as food flavorings and seasonings to improve the flavor, but they may also be used as traditional medicines once that their high nutritional and functional value is recognized, with known positive health effects.

The identification and quantification of spices and herbs properties involve a range of different analytical methods and techniques possible and the choice of methods to use is critical.

An approach for rapid characterization can be performed consulting a number of Standard Analytical Methodologies available in various informative documents as:

- European Spice Association quality specifications
- ASTA (American Spice Trade Association) – universally adopted manual for the assessment of quality of spices
- ISO (International Organization for Standardization)
- BSI (British Standards Institution)
- BIS (Bureau of Indian Standards)
- Others
    - Scientifically Valid Analytical Methods (Published Journal Articles)
    - Herbal Pharmacopeias

<u>Sampling</u>

Analysis starts with sampling. For trace analysis, incorrect sampling can represent a major source of errors. Sampling has to be adjusted to the different type of matrix and has to be performed in order to represent the herb or spice under analysis.

The quality of the spices and herbs is assessed by intrinsic and extrinsic characteristics. The former consists of chemical quality while the latter emphasizes the physical quality. This includes the appearance, shape, texture, color, presence or absence of extraneous things.

General analytical methods for spices and herbs characterization

- Sieve analysis
- Density
- Moisture content
- Fibers
- Total ash
- Antioxidant capacity
- Total phenolic compounds

Determination of Essential Minerals

a) Digestion for mineral analysis

b) Flame photometric determination: potassium, calcium and sodium

c) Spectrophotometric determination: phosphorus

d) Atomic absorption spectrophotometeric determination: Iron, zinc, manganese, cupper and magnesium

All of these parameters are usually determined as described by A.O.A.C methods

2.1. EXTRACTION AND CHARACTERIZATION OF NUTRITIONAL AND FUNCTIONAL INGREDIENTS

Spices and herbs provide protein, fiber, volatile components, vitamins, minerals and phytochemicals. One of the most important steps for the determination of phenolic compounds is their extraction, and in the case of the spices and herbs, these must be ground, and homogenized, subsequently using a solvent for extraction, and it was found that methanol was the most effective when used to thyme, sage and marjoram. The technique HPLC - High Performance Liquid Chromatography, is considered the most reliable and simple technique for the determination of phenolic compounds in foods and frequently used in the context of herbs. The fingerprint HPLC technique allows a kind of chemical compounds (to separate, identify and quantify each) and can thus be used as a means of quality control and consistency between different vendors of herbs a company for example.

Modern techniques use chromatography to separate the analytes and identify them with different detectors. Commonly used modern techniques include gas chromatography (GC), high performance liquid chromatography (HPLC) and ultra-high performance liquid chromatography (UHPLC). Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) has been recognized as a powerful analytical tool with its high sensitivity, short run time and less use of toxic organic solvents used as mobile phase compared to reversed phase standalone HPLC coupled with Diode-Array Detector.

Detectors refer to photodiode array detector (PDA), evaporative light scattering detector (ELSD), fluorescence detector, mass spectrometer, and electro chemical detector.

Infrared spectroscopy (IR) and Ultraviolet-visible spectroscopy (UV-vis) are used as analytical tools in the analysis of herbal samples.

IR/UV-vis methods can analyze samples that contain pure or very simple component.

IR/UV-vis methods can be used for quantitative analysis

Extracts preparation

Hydrophilic extractions are performed by homogenizing 5 g of the sample in 50 mL of methanol in water at the concentration of 800 mL/L. Homogenization is performed at 24000 rpm for 30 sec. The slurry is then centrifuged at 4000 ×*g* at 4 °C for 10 min, the supernatant filtered through a 0.45-µm cellulose acetate filter, the volume of the filtrate recorded and used for the assessment of total activities and quantification of individual compounds. A 10-mL aliquot of the extract is evaporated to dryness in a speed-vacuum evaporator at 30 °C and the residue dissolved in 3 mL of methanol. The extract is then filtered through a 0.45-µm cellulose acetate filter and analysed by HPLC-DAD.

Analysis of antioxidant activity

The free radical-scavenging activity can be determined by ABTS radical cation decolourization assay (Giao, Gonzalez-Sanjose, Rivero-Perez, Pereira, Pintado, & Malcata, 2007). ABTS is produced by reacting ABTS aqueous solution with potassium persulfate. The $ABTS^+$ solution is diluted with redistilled water to an absorbance of 0.700±0.02 at 734 nm and equilibrated at 23 °C. After addition of 1.0 mL of diluted $ABTS^+$ solution to 10 µL of polyphenolic extracts, the absorbance reading is exactly 6 min after initial mixing. All determinations are performed in triplicate and the results are expressed as mg ascorbic acid equivalent/g through the use of a calibration curve in the adequate range for each spice or herb.

Analysis of total phenolic compounds

The concentration of total phenolic compounds is determined colorimetrically by the Folin-Ciocalteu method (Singleton & Rossi, 1965). Hydrophilic extract (50 µL) are mixed with 50 µL of Folin-Ciocalteu reagent, 1 mL sodium carbonate solution (75 g/L) and 1.4 mL of ultra-pure water. After 1 h in darkness at room temperature, the absorbance is measured at 750 nm in a Shimadzu 1240 UV-visible spectrophotometer. Gallic acid monohydrate (0.015-1.00 mg/mL) is used as standard for calibration and construction of a linear regression line and water as blank. The total phenolics content is calculated as gallic acid equivalents in mg/g.

Identification and quantification of phenolics

Qualitative and quantitative profiles of phenolic COMPOUNDS can be determined by HPLC-DAD. Separation is performed in a reverse phase C18 column (250 x 4.6 mm i.d., 5 µm particle size and 125 Å pore size) with a guard column containing the same stationary phase. Chromatographic separation of phenolic compounds is carried out with a solvent A - formic acid, water and methanol (92.5:5:2.5) - and solvent B - methanol and water (94:6) - under the following conditions: linear gradient starting at 0 to 10 % solvent B in 10 min at 0.5 mL/min, 10 to 30 % in 40 min at 0.65 mL/min, 30 to 50% in 20 min at 0.75mL/min and from 50 to 0 % in 10 min at 1 mL/min. Injection volume is 20 µL. Detection is achieved by a diode array detector at wavelengths ranging from 200 to 600 nm in 2 nm intervals. Absorbance is measured at 280 nm (flavan-3-ols) and 320 nm (cinnamic acids).

Retention times and spectra of compounds are analysed by comparison with pure standards and quantification performed by the calibration curves and expressed as micrograms per gram of fresh biomass.

2.1.1. EFFECT OF HARVEST, PROCESSING AND STORAGE

The harvesting of herbs is one factor that influences the quality of the final product, since the time they are harvested determines the amount and type of constituents that vary during the year and, for some of the constituents, even along the day, verifying that generally for herbs the essential oil content is higher in the early hours of the day. Furthermore, the age of the plant also influences the aromatic constituents. The main reasons for a poor quality product are: harvest the plant when it is not yet mature and inefficient irrigation systems where there with a high risk of moisture retention and microbiological contamination. Immediately after harvest, it is necessary to take some precautions so that the plant does not undergo changes before being packaged or before it is subjected to a transformation process that increases its lifetime.

After harvesting, so as to prolong the life of the plant and to preserve its constituents, it is usually carried out the drying of herbs. This process has been evolving and can be performed in several ways, from sun drying (outdoors), the use of a well-ventilated shaded space; using a drying oven or tunnel, by continuous processing with hot air; using infrared rays or by oven drying under vacuum. In processing the material, the most widely used process is the hot air drying in dryers, with a recommended temperature of 20 to 40 ° C for leaves and flowering parts, usually conducted so that the products do not stay with an amount of water exceeding 10%. The loss of volatile compounds during drying herbs is higher for temperatures from 50 ° C and higher. The temperature which best preserves the volatile compounds from herbs is 40 ° C ("Encyclopedia of Food Sciences and Nutrition," 2003), since drying with high temperatures causes significant loss of flavor and aroma due to the loss of volatile compounds. Advances in technology have allowed to reach new heights in the process of drying herbs, by irradiation. The irradiation is a processing technique without the use of temperature, which ensures the elimination of pathogenic microorganisms and parasites, preserves food product and does not alter the organoleptic and nutritional characteristics of the food. Currently irradiation of herbs is used at industrial level with the decontamination purpose, complemented with other preservation process such as drying to dry herbs. After drying and before storage for subsequent packaging, it is necessary to sieve the dried herbs in order to ensure that they are of better quality, thus excluding foreign matter, dust and even stems.

It is common to process herbs milling through sieves to obtain a specific size. Grinding the herbs makes easier their addition to food and aids in the transmission of flavors and aromas. When herbs are in the form of powder it becomes more susceptible to changes, it increases its exposed surface and thus increases the losses of volatile compounds and oxidation phenomena occurs.

After drying, there are factors that must be taken into account so that there is correct conservation of the processed product, namely: light, temperature and humidity of the storage environment. Sun exposure causes rapid color change of herbs, some losing their characteristic bright green, and the ultraviolet rays factor can be a catalyst for changes of herbs constituents. On the other hand, the temperature also causes changes in the dried herb, and even more rapid when the temperature is higher, verifying significant losses of volatile compounds; therefore, it is recommended that spices and herbs are not conserved in temperatures exceeding 20 ° C. In turn, the moisture potentiates the enzyme activity and the likelihood of developing fungi, yeasts and other microorganisms, thus the recommended conservation is in environments with relative humidity levels from 40 to 60%. Furthermore, warehouses where the herbs are stored must have certain characteristics with extend to other companies in the food industry, in particular the control of pests and controlled ventilation, essential for these types of products.

During storage, spices and herbs components may be subjected to certain conditions that profoundly affect their properties. Phenolic antioxidants can be oxidized, producing free radicals or quinones that easily form polymers, which antioxidant activity may be greater or slower than that of the original form.

As already referred, phenolic antioxidants are present in spices and herbs as glycosides. During processing, hydrolysis splits flavones into simpler compounds or cleaves off phenols bound onto more complicated phenolic compounds. However, the reaction can also proceed in the opposite direction, leading to the formation of glycosides or condensed phenols. Therefore, the antioxidant capacity may be affected or not by processing.

During boiling, food is placed in boiling water at a temperature of approximately 90-110 °C. Some losses of antioxidant volatiles are caused by distillation with water vapour. Also, losses of ascorbic acid occur. Antioxidants bound by glycosides are partially hydrolyzed, but the activity of glycosides and free phenols is mostly maintained. Flavonoids and other phenolic compounds content is increased by their liberation from insoluble complexes. Microwave heating, as a faster process, reduces the losses when compared with conventional heating.

Frying leads to high phenolic compounds losses once that heating occurs in the presence of oxygen. Therefore and in all cases, is recommended that spices and herbs are added to the foods after processing.

Storage

Phenolic antioxidants are easily oxidized during storage. Oxidation increases the reactivity of antioxidants, as additional free radicals react with natural phenolic compounds in food, sulphur aminoacids bound in proteins and carotenes and other substances may be destroyed.

Room temperature, by itself, does not change significantly the antioxidant potential of spices and herbs, especially if enzymes are already inactivated. Storage in refrigerated room reduces the changes even further.

The preservation of the final product, aromatic spices and herbs with the desired particle size, is also affected by the type of package or container in which these products reach the consumer. Since the glass to plastic and paper/cardboard, there are several packages available in the market, with variable packing material: the glass is waterproof, while the remaining materials allow gas exchange between the outside and spices and herbs. The plastic containers and cardboard should be avoided, particularly when the product is powder, due to changes in the herbal constituents. It is noted that a good type of packaging are the aluminum foil pouches, however these are expensive and so are less used by the industry. The shelf life of processed spices and herbs is determined according to the parameters that lead to change, and generally can range from 1 and a half to three years. However, continuous monitoring is required, since the conservation mode will affect the expiration date.

2.1.2. BIOLOGICAL ACTIVITY AND TOXICOLOGY

Many spices are known to have medicinal properties, such as antioxidant activity, digestive stimulant action, anti-inflammatory, antimicrobial, hypolipidemic, antimutagenic, anticarcinogenic potential, etc. Herbs and spices bioactive compounds content is closely associated to these products beneficial impact on health. In the search for phytochemicals that are beneficial for human health, polyphenols are one of the most relevant targets, due to their biological activity. Moreover, many volatiles characterizing spices and herbs possess relevant biological activities in addition to their flavor (antibacterial, antiviral, antifungal, or toxic).

In recent decades, a number of phenolic substances were isolated from a variety of spice sources, including phenolic acids (e.g., gallic acid, caffeic acid, etc.), flavonoids (e.g., quercetin, rutin, myricetin, luteolin, naringenin, and silybin), phenolic diterpenes, and volatile oils. Noteworthy efforts have been performed in recent years to improve the knowledge of chemical composition of spices and herbs.

Despite the spices and herbs being used long for human consumption, are considered safe, their widespread use in industrial applications, which may involve the addition of much more than the quantities normally used, can increase human exposure to certain compounds and thus giving rise to toxicological effects not yet fully identified.

The dangers associated with the use of spices and herbs, their extracts and/or essential oils can be divided into two broad categories:

1. hazards arising from their chemical or microbiological contamination;
2. Dangers of its natural constituents.

The first category may meet heavy metals (cadmium, lead, mercury, arsenic, etc.), pesticides, dioxins and other polyaromatic hydrocarbons persistent organic pollutants which can contaminate the production areas and eventually be incorporated in the vegetable matrix. This group may still arise various microbiological hazards (bacteria, microbial toxins and mycotoxins) when the hygiene conditions in the production of these plants or their extracts are not properly respected.

In the second category are naturally occurring compounds in plants, whose considerable increase in the consumption and especially the increased consumption in the form of concentrated extracts and essential oils, may, in some way, present a risk to consumer health. In this case, you may need to further study what are the possible effects on human health resulting from the very high exposure to these plants, their extracts and essential oils.

Following, a number of spices and culinary herbs biological activity and some toxicity risks:

Saffron

In the case of saffron, which is one of spices known, cultivated and appreciated since ancient times throughout the Mediterranean basin, has a preventive effect on the skin cancer, stomach, colon and oral cancer. It also acts as quimioprotetor in inhibiting the formation of metastases in breast cancers. It has been referred as fluidizing and reducing blood cholesterol levels. However, its excessive consumption can be detrimental, to the extent that is considered abortifacient, hemorrhagic and may cause dizziness.

Basil

Basil seems to exert a preventive effect in skin and stomach cancer, inhibition of enzymes associated with diabetes, also having an anti-aging effect due to high levels of polyphenols. On the other hand, may moreover have positive effects on blood pressure and improve renal function. When consumed in tea, basil clears throat and lungs. It should not, however, be consumed in excess by pregnant women, since it can cause irritability.

Rosemary

Rosemary is accepted as to protect against breast cancer, hypertension and diabetes, and have an anti-inflammatory character. It is indicated for the treatment of headaches, nasal obstruction, poor digestion and loss of appetite, and even flatulence. It has a healing effect, anti-arthritic, analgesic, antidepressant, antibiotic and antirheumatic, and its essential oil is generally used as a mental stimulant. However, it should be avoided by hypertensive patients with prostate problems or diarrhea, gastritis, gastroduodenal ulcers, Crohn's disease patients, Parkinson's disease or irritable bowel syndrome as well

as other neurological conditions. Generally, consumption of high doses can cause toxicity and gastrointestinal irritation and skin.

Lemongrass

In the case of lemongrass, its protection is more restricted to colon cancer, but also plays an important role in combating flatulence, aiding in the digestion process and acting also as a sedative. Despite its benefits, this plant should be avoided by patients who are taking medication for thyroid or suffering from low pressure. In the case of the essential oil, 2 g intake can cause toxic manifestations, it can cause drowsiness, bradycardia and hypotension.

Mint

The mint is regarded as reducing the incidence of tumor and its spread in the case of the lungs. It acts even as antiseptic, soothing and stimulating bile secretion and digestive juices. Despite all its benefits, the mint should be avoided by pregnant or when breastfeeding and patients with ulcer. Furthermore, the plant can contribute to side effects such as changes in bowel habits, itching and rash in the skin.

Parsley

Parsley acts on the liver and the intestinal mucosa, and its use is often beneficial in the treatment of hypertension and diabetes, also having as many herbs, an anti-inflammatory potential. However, the use of this kind of herb should be avoided by pregnant women, patients with renal inflammation and patients with epilepsy. Excessive consumption can cause allergies, nausea and dizziness, hypotension, and some types of injuries such as kidney and liver.

Laurel

Containing various classes of flavonoids, it has antidiabetic effect and an anti-inflammatory potential, so that their consumption, when combined with a proper diet, results in good levels of cholesterol, triglycerides and glucose in the blood. Laurel leaves in infusion relieve flatulence and bloating, aiding digestion, and also intervene in problems such as arthritis. It should, however, not be used too much during pregnancy or lactation due to its active ingredients.

2.1.2.1. ANTIMICROBIAL EFFECT

The rising number and severity of poisoning outbreaks worldwide has increased public awareness about food safety issues. Also, the excessive use of chemical preservatives, many of which are believed to have negative impacts on health as well as having residual toxicity, has increased consumers concern. Currently, various spices and herbs are used as preservatives to assure that manufactured foods remain safe and unspoiled. Essential oils from edible and medicinal plants, spices and culinary herbs, are of great interest since they constitute a class of very potent and natural antimicrobial agents.

The antimicrobial activities of spices and herbs are multi fold and depend upon the phytoconstituents, concentration of phytoconstituents/phytochemicals and their bioactive principles, as well as antagonist actions. These phytochemicals include flavonoids, steroids, carotenoids, anthocyanins, quinones, glycosides, coumarins, alkaloids, saponins, tannins, phenolic acids and others. The individual components of the spices and herbs can be extracted from the plant or synthetically manufactured, have been found to have a wide range of antimicrobial properties, for example, against bacteria, fungi and mycobacteria.

Possible modes of antimicrobial action:

- At low concentrations, active components affect the activity of enzymes associated with energy production, while in higher concentrations they cause the precipitation of proteins;
- Impairment of various enzyme systems – deleterious effects on cellular membranes with pH and electrical potential changes, leakage of specific ions

- Impairment of respiratory activity of different bacteria and yeasts
- Changes in solubility of trace elements – for example, iron, an enzyme co-factor that permits their oxygenation
- Interactions between the antimicrobial constituents and the bacterial membrane components (proteins).

2.1.2.2. ANTICARCINOGENIC EFFECT

Spices and herbs may be a key to determining the balance between pro- and anticancer factors that regulate risk and tumor behavior. The ability of spices and herbs to serve as inhibitors of carcinogen bioactivation, decrease free radical formation, suppress cell division and promote apoptosis in cancerous cells, suppress microbial growth, and regulate inflammation and immunocompetence are plausible mechanisms by which selected spices and herbs may promote health and disease resistance. A range of bioactive compounds in spices and herbs have been studied for anticarcinogenic properties in animals, but the challenge lies in integrating this knowledge to ascertain whether any effects can be observed in humans, and within defined cuisines. As yet, there are no data indicating that herbs and spices have an anticarcinogenic effect in humans, but there are several in-vitro studies and rodent in-vivo studies suggesting that certain herbs and spices may have a chemopreventive effect against the early initiating stages of cancer. Herbs may act through several mechanisms to provide protection against cancer. Certain phytochemicals from herbs or herb extracts have been shown to inhibit one or more of the stages of the cancer process (ie, initiation, promotion, growth and metastases). Inhibition of phase I (procarcinogen activation) and induction of phase II (carcinogen deactivation) metabolic enzymes by herbal products may account for some of the preventive effects against the induction of gene or chromosomal mutations that may initiate cancer. For example, diallyl sulfide, a compound in garlic, is an efficient inhibitor of the phase I enzyme cytochrome P450 (CYP)3 IIE1 and significantly increases a variety of phase II enzymes, including glutathione S-transferase, quinone reductase and uridine diphosphate-glucuronosyltransferase, which are responsible for the detoxification of carcinogens.

Herbs may also protect against oxidative stress and inflammation, both of which are risk factors for cancer initiation and promotion as well as other pathological conditions. An imbalance between the generation of reactive oxygen species (eg, hydroxyl radical and superoxide radical anion) and cellular antioxidant capacity leads to a state of oxidative stress. Herbs and spices contain several natural water-soluble phenolic acids and flavonoids, such as caffeic acid and quercetin, that can scavenge reactive oxygen species, as well as containing lipid-soluble compounds such as tocopherols, carotenoids and sterols that may protect against the generation of genotoxic lipid peroxidation products, such as trans-4-hydroxy-2-nonenal.

Herbs and spices (or their fractions and constituents) with known anticarcinogenic effects in animal models of cancer include turmeric, basil, rosemary, mint and lemon grass, but there are no published reports on potential chemopreventive effects against cancer for other common spices such as thyme, coriander and dill. Turmeric has been widely used as a spice and colouring agent in foods. Recently, turmeric was found to have chemopreventive effects against cancers of the skin, forestomach, liver and colon, and oral cancer in mice.

2.1.2.3. ANTIOXIDANT EFFECT

Recently, interest has increased considerably in natural occurring antioxidants for use in foods as replacements for synthetic antioxidants. It is well known that some spices and herbs have potent antioxidant properties. Natural antioxidants can protect the human body from free radicals and could retard the progress of many chronic diseases. Another important role of natural antioxidants in spices and herbs is their effect on the reduction of lipid oxidation in foods, helping maintaining nutritional quality and reducing wastages and losses.

The most effective antioxidants found in spices and herbs are phenolic compounds, possessing at least two hydroxylic groups, in the ortho or para positions, as caffeic acid or most flavones or catechins. Some volatile components also have antioxidant activities, such as eugenol.

2.2. EXTRACTION AND CHARACTERIZATION OF ESSENTIAL OILS

Essential oils are liquid preparations produced from plant materials (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots) of temperate to warm countries, like Mediterranean and tropical areas. Only few of them are solid or resinous at room temperature; they are limpid, soluble in lipids or in organic solvents, with a generally lower density than that of water, with a pale yellow to emerald green or blue to dark brownish red color.

Each spice or herb is characterized by a peculiar quali-quantitative composition for its essential oil and all of those oils contain compounds with established biological activity.

The essential oils may be extracted from the plant using various methods such as distillation, hydrodistillation or by vapor entrainment, solvent extraction, cold pressing and extraction with liquid carbon dioxide. Once extracted, the essential oil is separated from the aqueous phase by processes that should not compromise its original composition. The organs of the plant and its stages of development and maturation are the determinants of oil composition.

Essential oils are mixtures of 20–60 components at quite different concentrations, with some compounds at fairly high concentrations (20–70%), and others in trace amounts. The components at high concentrations (terpenes, terpenoids, molecules with an aromatic ring) play a major role in the antimicrobial/biological effect. Some important compounds are mono and sesquiterpenes, carbohydrates, phenols, alcohols, ethers, aldehydes, and ketones. Phenolic compounds have also been recognized as bioactive components. Essential oils with aldehydes or phenols as major components (cinnamaldehyde, citral, carvacrol, eugenol, or thymol) are the most effective, followed by the ones containing terpene alcohols. Still, the extraction in each species requires testing purposes, so as to determine the parameters to obtain an essential oil as pure as possible. Essential oils are constituted by volatile components, insoluble in water but soluble in organic solvents and lipids, and each compound in a specific ratio gives the unique characteristic of each essential oil.

Analysis of the chemical composition of essential oils obtained from plants can be performed by gas chromatography and/or mass spectrometry. An essential oil may comprise more than 60 individual volatile components.

Essential oils extraction methods (Asbahani et al; 2015)

Hydrodistillation

This method is the most simple and old that is used for the extraction of essential oils. The plant material is immersed directly in the water inside the alembic and the whole is brought to boiling. The extraction device includes a source of heating surmounted by a vessel (alembic) in which we could put plant material and water. The set up comprises also a condenser and a decanter to collect the condensate and to separate the essential oils from water, respectively. The principle of extraction is based on the azeotropic distillation. In fact, at atmospheric pressure and during extraction process (heating), water and essential oil molecules form a heterogeneous mixture which attained its boiling temperature at a lower point close to 100 °C while for essential oils components this point is very high. The mixture is then distilled simultaneously as if they were a single compound. This is referred as co-distillation in the presence of vapors of water as solvent drive. The advantage of water is that it is immiscible with the majority of the terpenic molecules of essential oils and thus, after condensation, they could be easily separated from water by simple decantation. The hydrodistillation by Clevenger system is recommended by the third edition of the European Pharmacopoeia for the determination of essential oil yields. It allows the recycling of the condensates through a cohobage system. This method is suitable for the extraction of petals and flower (i.e. petals of rose) as it avoids compacting and clumping of plant material during extraction. The hydrodistillation has, however, several drawbacks: (i) long extraction time (3–6 h; 24 h for the rose petals), (ii) artifacts and chemical alterations of terpenic molecules by prolonged contact with boiling water (hydrolysis, cyclization…) and (iii) overheating and loss of some polar molecules in the water extraction. An optimized variant of this technique, the turbodistillation allows to obtain high yields by recycling the aromatic water. It reduces distillation time thanks to the presence of turbines (allow fragmentation and agitation). In addition, it enables almost complete recovery of essential oils present in the vapor through the plate column. In industrial scale, this method is still used for several reasons: (i) simplicity of installations (does not require expensive equipment), (ii) easiness of method implementing and (iii) its selectivity.

Entrainment by water steam

It is one of the official methods for the obtaining of essential oils. It is a widely used method for their extraction and it is based on the same principle as hydrodistillation with the difference that there is no direct contact between plant and water. Extraction duration is shortened thus reducing chemical alterations. There are other variants:

- Vapor hydrodistillation

Extraction is done within the alembic except that there is a system of perforated plate or grid that maintains the plant suspended above the base of the still containing water which avoids their direct contact. The extraction is done by injection of water vapors which cross plant matter from the bottom up and carries the volatile materials. Artifacts are minimized. The extraction time is reduced as well as the loss of polar molecules.

- Vapor distillation

This method has the same principles and advantages as the vapor-hydrodistillation, but the generation of vapors occurs outside of the distillation alembic. The steam can then be saturated or superheated; at slightly above atmospheric pressure, the steam is introduced into the lower part of the extractor and therefore passes through the raw material charge. This technique avoids some artifacts compared to hydrodistillation.

- Hydrodiffusion

This is a particular case of vapor-distillation where vapors' flow occurs downward. It is also called down hydrodiffusion or hydrodiffusion and gravity.

Organic solvent extraction

The plant material is macerated in an organic solvent; the extract is concentrated by removing the solvent under reduced pressure. This technique avoids alterations and chemical artifacts by cold extraction compared to hydrodistillation. Indeed, during hydrodistillation, the immersion of plant material in the bowling water causes water solubilisation of some fragrance constituents and reduces medium pH to 4–7 (sometimes less than 4 for some fruits). The constituents of the original plant species are subjected to the combined effects of heat and acid, and are subject to chemical modifications (hydrolysis, deprotonations, hydrations and cyclizations). Obtained essential oils differ significantly from the original essence, especially, if boiling is long, and pH is low. In another hand, extracts obtained by organic solvent contain residues that pollutes the foods and fragrances to which they are added. This compromises the safety of products extracted by this technique. Thus, it is impossible to use them for food or pharmaceutical applications. These disadvantages could be avoided by using a combination technology of organic solvent with low boiling point (e.g. *n*-pentane) and steam distillation process.

Cold pressing

Cold pressing is the traditional method to extract essential oils from citrus fruit zest. During extraction, oil sacs break and release volatile oils which are localized in the external part of the mesocarpe (sacs oils or oil glands). This oil is removed mechanically by cold pressing yielding a watery emulsion. Oil is recovered subsequently by centrifugation. In this case we obtain the vegetable essence of citrus zest which is used in food and pharmaceutical industries and as flavoring ingredients or additives (food industry, cosmetics and some home care products).

Supercritical fluid extraction (SCFE)

For fluids, the supercritical state is reached at well defined conditions: critical pressure (Pc) and temperature (Tc). Fluids could then exhibit very interesting properties: (i) low viscosity, (ii) high diffusivity, (iii) density close to that of liquids. Carbon dioxide is generally the most widely used solvent for essential oils extraction because of its numerous advantages: (i) critical point is easily reached (low critical pressure, Pc: 72.9 atm, and temperature, Tc: 31.2 °C), (ii) unaggressive for thermolabile molecules of the plant essence; (iii) it is chemically inert and non-toxic, (iv) non flammable, (v) available in high purity at relatively low cost, (vi) easy elimination of its traces from the obtained extract by simple depression and (vii) its polarity similar to pentane which makes it suitable for extraction of lipophilic compounds. The principle is based on the use and recycling of fluid in repeated steps of compression/depression. By highly compressing and heating, CO_2 reaches the supercritical state. It passes through the raw plant material and loaded volatile matter and plant extracts. This is followed by a depression step: the extract is routed to one or more separators, where the CO_2 is gradually decompressed (thus losing its solvent power) to separate the obtained extract from the fluid. The latter could be turned into a released gas and then could be recycled. The use of this technique for essential oils extraction has increased in the last two decades. The only one obstacle to its development is the high cost of the equipments, their installations and their maintenance operations. Supercritical extracts proved to be of superior quality, with better functional and biological activities in comparison with extracts produced by hydro-distillation or with liquid solvents. Furthermore, some studies showed better antibacterial and antifungal properties for the supercritical product.

Ultrasound assisted extraction

Ultrasound allows intensification and selective of essential oils extraction by accelerating their release from plant material when used in combination with other techniques (hydrodistillation and solvent extraction). The vegetable raw material is immersed in water or solvent and at the same time it is subjected to the action of ultrasound. The used ultrasonic waves have a frequency of 20 kHz–1 MH. This induces mechanical vibration of the walls and membranes of plant extract inducing a rapid release of essential oils droplets. The extraction mechanism involves two types of phenomena: diffusion trough the cell walls and washing out the cell content once the walls are broken. In fact, essential oils are stored in the plant in specific internal or external structures in the form of glands filled with droplets. Their skins are very thin that can be easily destroyed by sonication (in the case of external structures). For internal ones, the milling degree of plant material plays an important role in the obtained yield. It is obvious that reducing the size of plant material will increase the number of cells exposed to ultrasonically induced cavitations.

Microwave assisted extraction (MAE)

Microwaves are electromagnetic based waves with frequency between 300 MHz and 30 GHz and a wavelength between 1 cm and 1 m. The commonly used frequency is 2450 MHz which corresponds to a wavelength of 12.2 cm. The use of MAE evolved with the development of the green extraction concept and the need for new energy saving extraction methods. More attention has been paid to the application of microwave dielectric heating for essential oils extraction. Starting from compressed air microwave distillation (CAMD) and vacuum microwave hydrodistillation (VMHD), innovation in the microwave assisted extraction (MAE) led to the development of a large number of variants such as microwave assisted hydrodistillation, solvent free microwave extraction (SFME), microwave-accelerated steam distillation (MASD), microwave steam distillation, microwave hydrodiffusion and gravity (MHG) and portable microwave assisted extraction (PMAE). The MAE became rapidly one of the most potent extraction methods and one of the upcoming and promising techniques. It offers high reproducibility in shorter times, simplified manipulation, reduced solvent consumption and lower energy input.

Determination of yield in essential oils

Percentage of volatile oil extracted is calculated on fresh and dry weight basis in replicate distillations from the tested spices and herbs according to the following Equation:

$$Volatile\ oil\ (\%) = (weight\ of\ the\ volatile\ oil\ recovered \div weight\ of\ the\ sample) \times 100$$

The volatile oil is removed from the receiver with ether and dried overnight using anhydrous sodium sulphate before removing the ether. The obtained volatile oil is stored in the dark at a temperature of −18˚C until requiring for analysis.

Gas chromatography of essential oils

A gas chromatograph is a chemical analysis instrument used to separate and identify individual constituents found within a given essential oil. Each chemical constituent of an essential oil will pass through the gas chromatograph instrument and different times and speeds. As each chemical is registered it will produce some type of peak, from very short to very tall. A gas chromatography report reveals the peaks of different chemical constituents within a given oil, it does not, however, name the specific chemical constituent (e.g. linalol), for this a mass spectrometry must be used. Mass spectrometry is a technique which allows for the detection of compounds (chemical constituents) by separating ions by their unique mass. Mass spectrometry is utilized to identify specific compounds registered on the gas chromatography report. A typical mass spectrometer has three basic parts: an ion source, a mass analyzer, and a detector. Different molecules have different masses, and this fact is used to determine what molecules are present in a sample. An individual trained in reading GC/MS data will then clearly identify the exact constituents and their quantity (e.g. 5% linalol, 25% camphor, and so on) present within a given essential oil sample.

Example Apparatus and conditions for separation

Volatile compounds in essential oils of dried spices and herbs are identified by comparison with kovats gas chromatographic retention index and by the mass spectral fragmentation pattern of each GC component compared with authentic compounds. A gas chromatograph equipped with a DB5 capillary column (30 m × 0.25 mm i.d. × 0.25 µm df.), FID detector is used. The analysis is carried out under the following conditions: injector temperature 200˚C and detector temperature 250˚C. The column was programmed from 35˚C to 220˚C at 30˚C/min and held for 40 min. The helium carrier gas flow rate was 29 cm/sec. Injections are in the split less mode.

2) Identification and quantitation

Kovat's indices are determined by co-injection of the sample with a solution containing homologous series of n-hydrocarbons (C6-C26) under the same conditions as described above. The separated components are identified by matching with N1ST mass-spectral library data, and by comparison of Kovat's indices with those of authentic components and with published data. The quantitative determination is carried out based on peak area integration.

2.2.1. VALIDATION OF BIOLOGICAL ACTIVITY AND TOXICOLOGY

The main essential oil constituents possess significant antibacterial and antioxidant properties, mainly attributed to phenol monoterpenes content. Purified compounds derived from essential oils such as carvacrol, eugenol, linalool, and thymol inhibit a variety of micro-organisms. The component with the widest spectrum is thymol followed by carvacrol. Carvacrol has been characterized as an inhibitor of growth of different pathogens and was shown to have a bactericidal effect towards *Salmonella* in pieces of fish stored at 4°C and causes a sharp decrease of the toxin production by *Bacillus cereus*.

In general, levels of essential oils and their compounds necessary to inhibit microbial growth are higher in foods than in culture media. This is due to interactions between phenolic compounds and the food matrix.

The assay methods reported in the literature use various measurements to register antimicrobial effects (Peter, 2012):

- the bacterial growth inhibition zone around a paper disc containing the compound, or mixture, tested, on various substrates
- the inhibition of bacterial growth on an agar medium when the teste compound, or mixture, is diffused in the agar
- the optical density changes of a growth medium to which inoculum and antimicrobial agent have been added

The production of bioactive spices and culinary herbs requires the development of well-validated analytical methods to ensure their quality, as well as safety and efficacy within different batches. For that, the analytical test method validation is completed to ensure that an analytical methodology is selective, accurate, reproducible, and robust over the specified range in which an analyte is analyzed. For method validation, guidelines from the regulatory agencies provide a framework to perform such validations [2]. Essential criteria for the quality of natural compounds, pharmaceuticals, cosmetics, foods, and other products are ensured by method validation. Regarding phytotherapeutic agents development, a validated analytical method capable of analyzing natural complex matrices is required throughout all steps, which includes:

- the selection of a good plant cultivar and the determination of the best time of harvesting; - the determination of extraction conditions and drying process;
- the development of an adequate formulation able to deliver the active compounds;
- the quantification of analytes during the production processes;
- the analyses of the final product and determination of its shelf life;
- the quantification of the active compounds in biological matrices to follow up both pre-clinical and clinical assays, among others.

Biological profiling in terms of anti-microbial and antioxidative efficacies of the spices and herbs using selected bacteria and/or fungi following the established simple method(s) can be used as an "effective bioefficacy marker". These simple bioefficacy markers can became increasingly devoted to the identification of bioactive botanical products and their standardization.

Because of their complex chemical composition, often composed of more than 100 different compounds, essential oils have a broad biological and antimicrobial activity spectrum (antibacterial, antifungal, anti-moulds, antiviral, pest control, insect repellents). In the pharmaceutical field, essential oils are included in the composition of many dosage forms (capsules, ointments, creams, syrups, suppositories, aerosols and sprays).

Food industry also presents a growing demand for essential oils because of their important applications as food preservatives, innovation in food packaging and the fight against pathogens generating dangerous food poisoning (*Listeria monocytogenes*, *Salmonella typhimurium*, *Clostridium perfringens*, *Pseudomonasputida* and *staphylococcus aureus*).

Other applications include medical and technical textiles. In this case, encapsulation is the technique of choice in industries process as a means of imparting finishes and properties on textiles that were not possible or cost-effective using other technologies. In textiles, the major application of encapsulation is durable fragrances and skin softeners. Other applications include insect repellents, dyes, vitamins, antimicrobial agents, phase-change materials and medical applications, such as antibiotics, hormones and other drugs.

Essential oils are unstable and fragile volatile compounds. Consequently, they could be degraded easily (by oxidation, volatilization, heating, light) if they are not protected from external factors. Such protection could increase their action duration and provide a controlled release. Essential oils stability could be increased by encapsulation which has been also shown to improve the antibacterial activity of several antibiotics. Major limitation is essential oils loss especially in techniques that include a heating or an evaporation step. In fact, high temperatures, UV light and oxidation could compromise the biological activity of fragile essential oils through volatilization or degradation of active ingredients. Formulation of essential oils as microcapsules or microspheres could also be used for controlling release of encapsulated essential oils. Various techniques have been successfully used to attain this purpose with interesting results. Many other advantages were obtained after loading essential oils in particles or liposomes such as, enhanced efficacy and sustained release.

2.2.2. ENCAPSULATION TO ASSURE CONTROLLED RELEASE AND PROTECTION

Within the wide range of available essential oils, a common need is the availability of natural extracts with a pleasant smell and/or taste, combined with a preservative action, aimed to avoid lipid deterioration, oxidation or spoilage by microorganisms. Those undesired phenomena induce the development of unpleasant off-flavours, create toxicity and severely affect shelf-life of products. The products quality used to be traditionally determined by physical, chemical and microbiological criteria however, soon it was realized that without sensory evaluation, the acceptability of a product cannot be determined.

Sensory quality of essential oils is characterized by attributes of appearance, odor/aroma/fragrance, consistency and texture and flavor (aromatics, chemical feeling, taste). Essential oils are perceived by senses individually and processed by the brain into a total impression of quality. The viscosity, colour, clarity and odour of an essential oil can help to identify a poor quality oil.

2.3. SENSORY AND AROMATIC AND FUNCTIONAL PROPERTIES ACCORDING: HARVEST SEASON AND TECHNOLOGY, PROCESSING AND STORAGE

The sensory, nutritional and functional properties of an essential oil is determined not only by the genetic component of the plant, but also by external factors, including environmental and geographical conditions in a region, as well as the harvesting and conservation techniques and the time of year when harvested.

It is in the interests of the grower, and the industry, to produce a high quality product that will attract a premium market price. Pre-harvest operations involve the preparation of the facilities for the harvest material, which will ensure the crop is stored and dried quickly under hygienic conditions. The main reasons for low quality product are harvesting the crop when it is not mature; poor drying systems where there is a high risk of moisture retention and microbial contamination (dirt floors); and frequent rain during the drying process, which upsets the drying process. There is need to have buildings or structures at the harvesting area or to have a common facility for drying and curing products. Many growers of spices use traditional methods and high moisture retention, microbial contamination, and contamination with extraneous matter are common processing problems. International sanitary and phytosanitary agreements define measures to be taken to protect against risks arising from additives, contaminants, toxins or disease causing organisms in food or foodstuffs. In particular, there are problems with mould, high moisture contents and aflatoxin contents. Difficulties in reducing these problems to a low level are due to poor weather conditions at harvest associated with low cost processing technology; poor storage facilities and small-scale production units. Poor storage facilities and unhygienic and improper storage methods also contribute to contamination with mammalian and other excreta, as well as moulds or other microbes. In order to overcome these problems completely, capital investment is necessary, particularly for mechanized handling after harvest.

Plants should be harvested during the optimal season or time period to ensure the production of plant materials and finished spice products and essential oils of the best possible quality. The time of harvest depends on the plant part to be used. Detailed information concerning the appropriate timing of harvest is often available in published standards, official monographs and major reference books. However, it is well known that the concentration of target constituents varies with the stage of plant growth and development. The best time for harvest should be determined according to the quality and quantity of target constituents.

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