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Erasmus+

INNOVATIVE INTEGRATED TRAINING IN HEALING PLANTS BUSINESS

IO3 - The Total Business Plants Training Material

Module No. 3

“Quality control of the final product (Medicinal plants)”

Unit 4: Laboratory techniques

- Summary

This unit offers an insight into the Laboratory techniques used to maintain the highest level of quality control. It explains thoroughly the concept of all these techniques and how helpful they are in order to make sure that the plant sample is safe to use and free from and toxic chemicals etc. These techniques also help in measuring the purity and also finding out all the ingredients in a sample.

- Learning outcome descriptors

By the end of this unit the trainee should be able to

1. Explain the different laboratory techniques
2. Explain what a DNA technique is
3. Decide which technique is best for the sample he has.

- General and transferable skills

1. Explain different DNA and laboratory techniques
2. Work independently
3. Organize tasks

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Methods for quality control of herbal medicines involve analytical inspection using chromatographic and spectroscopic techniques. They include TLC, HPLC, GC, ultraviolet (UV), Fourier transform infrared (FT-IR), atomic absorption spectroscopy (AAS), fluorimetry, near infrared (NIR), and the spectrophotometer. HPLC fingerprinting includes the recording of the chromatograms, the retention time of each individual peak, and the absorption spectra with different mobile phases. Similarly, gas–liquid chromatography (GLC) is used for generating the fingerprint profiles of volatile oils and fixed oils of herbal drugs. Furthermore, the recent approaches of applying hyphenated chromatography and spectrometry such as gas chromatography–mass spectrometry (GC–MS), capillary electrophoresis-diode array detection (CEDAD), high performance liquid chromatography–thin layer chromatography (HPTLC) and high performance liquid chromatography–mass spectroscopy (HPLC–MS), could provide the additional spectral information, which will be very helpful for the qualitative analysis and even for the on-line structural elucidation (4, 19, 20, 21).

There is also a plethora of DNA techniques that are being used as a supplement to methods and techniques of classical botany for plant identification, including RAPD, RFLP, ARMS, CAPS, AFLP, ISSR, hybridization and microarrays (22). A recent technological development is the authentication of medicinal plants by barcode DNA. This method is based on the detection of variable sites of the rDNA internal transcribed spacer (ITS). DNA barcoding provides a powerful tool for the authentication of plants, and is exquisitely suited for quality control of medicinal plants. Research that is currently conducted in this field focuses on the question, how many and which DNA fragments are necessary for the optimal discrimination of different species.

The following is a presentation of each of the aforementioned techniques and the ways they offer quality assurance of the final product.

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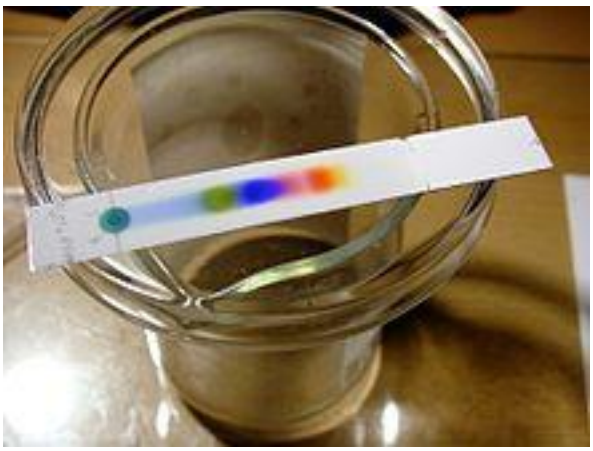
CHROMATOGRAPHIC/ SPECTROSCOPIC TECHNIQUES

TLC (Thin layer chromatography): Thin-layer chromatography (TLC) is a chromatography technique and it is used for the separation of non-volatile mixtures (23). It uses a sheet of either glass, plastic or aluminum foil that is covered with adsorbent material (silica gel or aluminum oxide) and this is called the stationary phase. Next, the sample is applied on the plate and then the mobile phase (a solvent or solvent mixture) is drawn up the plate through capillary action. The mixture is separated due to the fact that different analytes can ascend a TLC plate at different rates (24). It is important to know that the mobile and stationary phase have different properties and a mobile phase can also be a mixture and this enables any chemist to fine tune the properties of the mobile phase. This, as a result, can be used when performing quality control on mixtures of extracts of medicinal plants and their final products and is a relatively easy method of separating mixtures.



Separation of black ink on a TLC plate

HPLC: High-performance liquid chromatography, is a laboratory technique used on a mixture and it helps to separate, identify, and quantify each component. It utilizes pumps to pass a liquid solvent that is under pressure, containing the sample mixture through a column filled with a solid adsorbent material. It is known that each component that exists the sample, interacts slightly differently with the adsorbent material that is used each time and thus, different flow rates for the different components exist and this is leading to the separation of the components as they flow out the column (25). With this technique, the scientist has total control over operational parameters such as the flow rate of the mobile phase, buffer control of the mobile phase, column oven temperature, etc. In addition, HPLC software is capable of

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reporting precise and accurate results based on area counts of peaks and this can help on quality assurance of medicinal plants via identification of the elements in a mixture and separate them depending on quality.

An HPLC

GC: Gas chromatography (GC) is a chromatography technique used for separating and analyzing compounds that can be vaporized. Typical uses of GC include testing the purity of a particular substance (for example our final products of medicinal plants), or separating the different components of a mixture (the relative amounts of such components can also be determined, a useful ability when we need to determine foreign matter in a mixture). In some situations, GC may help in identifying a compound. GC can be used to prepare pure compounds from a mixture (26, 27). Gas chromatography is similar to column chromatography regarding its principles, but has several differences. First, the process of separating the compounds in a mixture is performed between a liquid stationary phase and a gas mobile phase, whereas in column chromatography the stationary phase is a solid and the mobile phase is a liquid. (Hence the full name of the procedure is "Gas–liquid chromatography", referring to the mobile and stationary phases, respectively.) Second, the column through which the gas phase passes is found in an oven where the temperature of the gas can be controlled, whereas column chromatography has no such temperature control (26).

Ultraviolet–visible spectroscopy: Ultraviolet–visible spectroscopy or ultraviolet-visible spectrophotometry (UV-Vis or UV/Vis) is a technique that yields results in the ultraviolet-visible

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spectrum. This means it uses light in the visible and adjacent spectra. The absorption or reflectance in the visible range can directly affect the color of the chemicals involved. In this region of the electromagnetic spectrum, atoms and molecules undergo electronic transitions. Absorption spectroscopy is complementary to fluorescence spectroscopy, because fluorescence is used for transitions from an excited state to the ground state, while absorption measures transitions from the ground state to an excited state (28). UV/Vis spectroscopy can be utilized for the quantitative determination of different analytes, for example, highly conjugated organic compounds, and biological macromolecules. Spectroscopic analysis is performed in solutions but solids and gases may also be studied.

Beckman DU640 UV/Vis spectrophotometer.

Fourier transform infrared spectroscopy: Fourier transform infrared spectroscopy (FTIR) is a technique which is used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas. The fact that an FTIR spectrometer collects high spectral resolution data over a wide spectral range simultaneously, confers a significant advantage over a dispersive spectrometer which measures intensity over a narrow range of wavelengths at a time. The goal of any technique relevant to absorption spectroscopy, is to measure how well a sample absorbs the light at each wavelength. This can be done through shining a monochromatic beam at a sample and then measure light absorption and then repeat these steps for each different wavelength.

Fourier transform spectroscopy can be used to obtain results in a less intuitive way. Rather than shining a monochromatic beam of light at the sample, this technique shines a beam containing many frequencies of light at once, and measures how much of that beam is absorbed by the sample. Next, the beam can be modified in order to contain different combinations of frequencies, giving a second data point. This process is repeated many times. Afterward, a

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computer takes all this data and works backward to infer what the absorption is at each wavelength (29).

AAS: Atomic absorption spectroscopy (AAS) refers to a spectroanalytical method that is utilized for the quantitative determination of many chemical elements by using the absorption of light by free atoms that exist in the gaseous state. In analytical chemistry, the technique is used to determine the concentration of a particular element (called the analyte) in a sample to be analyzed. AAS is able to determine over 70 different elements existing in a solution or directly in any solid sample that is used in pharmacology, biophysics and toxicology research. AAS is widely used in chemistry, on the analysis of biological fluids, tissues such as blood, saliva etc. It is also utilized in the analysis of the final drug product regarding the quantity of the catalyst that remains in it (30).

Flame atomic absorption spectroscopy instrument

Near-infrared spectroscopy: NIRS is a spectroscopic method that uses the near-infrared region of the electromagnetic spectrum (700 nm to 2500 nm). Typical applications include diagnostics and research. Numerous applications in other areas exist as well, such as pharmaceutical, food and agrochemical quality control, something that is of interest to us. Near-infrared spectroscopy is widely applied in agriculture for the determination of the quality of fruits, vegetables, eggs and dairy products, coffee, tea and spices. By utilizing NIRS one can quantify the compositions of those products in a reliable, fast, cheap and non-destructive way (31). So, it

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is very important to utilize NIRS for the determination of the quality of our final products and mixtures of final products regarding medicinal plants.

Spectrophotometer: A spectrophotometer is used for the measurement of either transmittance or reflectance of solutions and other forms of matter such as transparent or opaque solids, polished glass, or gases. Colorless biochemicals can be converted and be colored in order to be suitable for reactions that form chromogenic compounds and then perform a colorimetric analysis (32). Spectrophotometry is a technique often used for important measurements including those of enzyme activities, determinations of protein concentrations and determinations of enzymatic kinetic constants. Ultimately, when using a spectrophotometer we are able to determine, the substances present in a target sample and exactly how much is there, through calculations of the observed wavelengths, something helpful when the target is a mixture of medicinal plants or an extract as well.

GC - MS: Gas chromatography–mass spectrometry (GC-MS) is an analytical method that combines the features of gas-chromatography and mass spectrometry for the identification of different substances within any given sample (33). Of the many applications of GC-MS, some include drug detection, environmental analysis, and identification of unknown samples (useful when we examine a sample from an unknown plant or mixtures). What is even more interesting is that GC - MS can identify trace elements in materials thought to have disintegrated beyond identification through analysis and detection of even the tiniest amounts of a substance. The GC-MS machine is composed of two major blocks: the gas chromatograph and the mass spectrometer. As for the technique, it uses a capillary column which depends on the column's dimensions (length, diameter, film thickness) as well as the phase properties (e.g. 5% phenyl polysiloxane). Separation of the molecules while the sample travels through the length of the column will happen because of the different chemical properties that different molecules in a mixture have. Also, their relative affinity towards the stationary phase of the column is a factor for the separation. The column retains the molecules and then they elute at different times (this is called retention time). This is what allows the mass spectrometer to capture, deflect and detect the ionized molecules each one separately.

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Example of a GC-MS instrument

CEDAD: Capillary electrophoresis (CE) is a method that is performed in capillaries and in micro-fluidic and nano-fluidic channels. The term diode array detection (DAD) when used in capillary electrophoresis (CE) can effectively offer similar advantages as in HPLC, regarding single-wavelength detection. With an optimized optical system, DAD yields sensitivity comparable to that of single or variable wavelength detectors (34). CEDAD is useful when one needs the separation of elements of a mixture in order to next identify them, as in our case, the separation of mixtures of different elements.

HPTCL: High performance thin layer chromatography, is a form of thin layer chromatography that is enhanced with a number of improvements in order to increase the resolution and thus allow more accurate measurements(35). The procedure begins by using a plate that has the samples in it and then we load the first solvent. Then, we rotate the plate by 90° and then it is developed with a second solvent.

HPLC – MS: Liquid chromatography–mass spectrometry (LC-MS) refers to a technique used by analytical chemists and can combine the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry (MS). Systems that couple MS and chromatography are very popular in chemical analysis because the individual capabilities of each technique are enhanced synergistically. The advantage of using HPLC – MS is that while liquid chromatography separates mixtures with multiple components, mass spectrometry can provide is with the structural identities of each of the individual components

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with both a high molecular specificity but also sensitivity in detection. This is technique that it is usually called a “tandem technique” and it can be used to analyze biochemical, organic, and inorganic compounds that we commonly find in complex samples of environmental and biological origin. Therefore, LC-MS can be applied in a wide range of fields including biotechnology, environmental monitoring, pharmaceutical and agrochemical industries, something of great interest especially when dealing with medicinal plants (36, 37). In addition to the liquid chromatography and mass spectrometry devices, an LC-MS system can contain an interface that can efficiently transfer the separated components from the LC column into the MS ion source (37, 38). LC-MS is frequently used in drug development because it allows the quick confirmation of molecular weight and structure identification of the drug under investigation. These features speed up the process of generating, testing, and validating a discovery starting from a vast array of products with potential application. LC-MS can be applied as a technique for drug development and especially for in vivo drug screening, metabolite identification, impurity identification, quantitative bioanalysis, and quality control (39).

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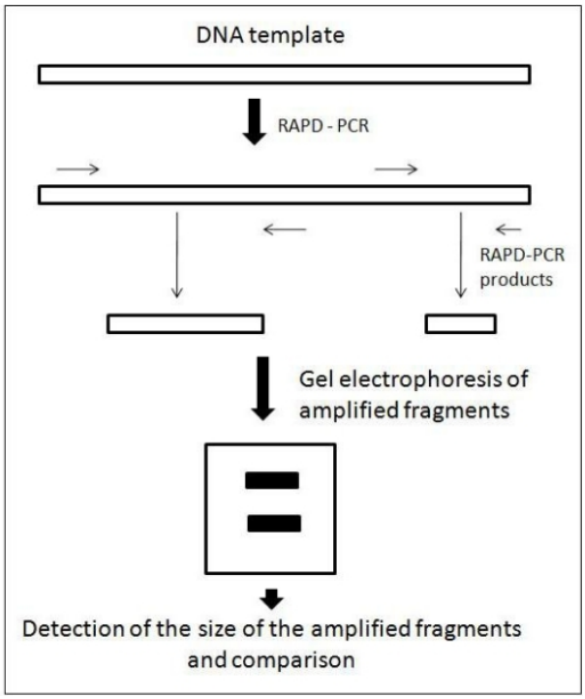
DNA TECHNIQUES

RAPD: RAPD means 'Random Amplification of Polymorphic DNA' (40) and it is a type of PCR reaction, but the amplified DNA segments are random and not specific. The main principle of the technique is the creation of several arbitrary and short primers (8–12 nucleotides), followed by the PCR using a large template of genomic DNA and hoping that some fragments will amplify. The resulting patterns can be resolved and we can obtain a semi – unique profile from this reaction. No knowledge of the DNA sequence of the targeted genome is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. RAPD is an inexpensive yet powerful typing and identifying method for many bacterial species. It can be used when we need to obtain soil for the creation of a medicinal drug and it needs to be bacteria free. The identification of the different strains can lead to the best strategy for getting rid of them.



The RAPD technique

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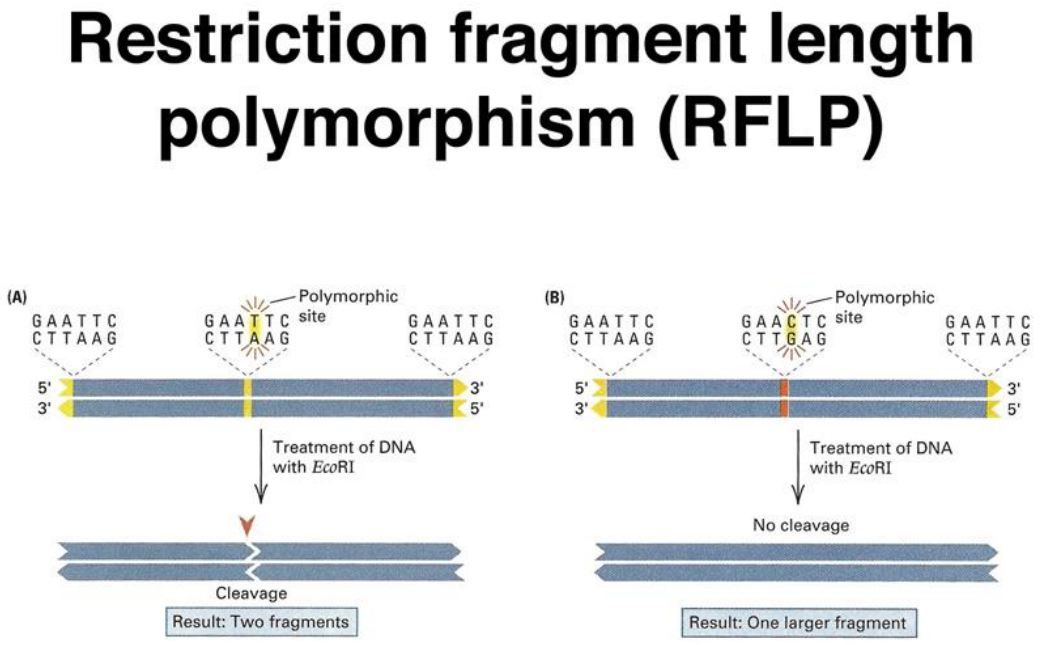
RFLP: In molecular biology, RFLP, is a technique that uses the variations that exist in homologous DNA sequences. When performing RFLP, the DNA sample must be broken into segments (digested) by known restriction enzymes. Then, those segments are separated according to their length by using gel electrophoresis. This technique is useful when we want to illustrate the differenced between certain DNA samples and also group them according to the digestion patterns. RFLP analysis has been for years the basis for early methods of genetic fingerprinting, useful in the identification of samples often retrieved from crime scenes etc, but also from environmental samples. This helps in the identification of certain rare plants and their byproducts.



The RFLP technique

ARMS: The amplification-refractory mutation system (ARMS) is a simple method used for the detection of mutations called SNPs (Single nucleotide polymorphisms) or very small deletions. The technique principle is based on the use of sequence-specific PCR primers that allow the amplification of the DNA only when the target allele is contained within the sample. After completion of the ARMS reaction, either the presence or absence of a PCR product is indicative of the presence or absence of the target allele. This can help in identification of mutated strains of bacteria of fungi that can be present in the final product and also be useful (for example the Bifidus Actiregularis bacteria).

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CAPS: The cleaved amplified polymorphic sequence or CAPS method is a technique in molecular biology for the analysis of genetic markers. It is an addition, or extension to the RFLP method, using polymerase chain reaction (PCR) for faster result analysis. Similar to RFLP, CAPS also exploits the fact that differences on individuals can offer new restriction enzyme sites or abolish existing ones. Then, those differences can be detected and analyzed. In the CAPS method, PCR amplification is mainly focused towards the altered restriction site, and the products digested with the restriction enzyme. When agarose or acrylamide gel electrophoresis is performed, the digested PCR products will give distinguishable patterns of bands.

AFLP: AFLP-PCR or just AFLP is a PCR-based technique that is used in genetics research, and in genetic engineering. The method is simple and it utilizes restriction enzymes in order to digest DNA and then, specific adaptors are ligated to the sticky ends that the enzymes have created. A small group of those fragments is selected and amplified. Selection is based on the use of primers that are complementary to the adaptor sequence, the restriction site sequence and a few nucleotides inside the restriction site fragments. After the amplification, the fragments can be separated and then visualized on polyacrylamide gels. The results obtained are referred to as presence – absence polymorphisms (41). The AFLP technology has the capability to detect various polymorphisms in different genomic regions simultaneously. It is also highly sensitive and reproducible. All of the above, make AFLP a suitable tool for the identification of genetic variation in various strains of plants, animals, fungi etc.

ISSR – PCR: ISSR (for inter-simple sequence repeat) is a general term for a genome region between microsatellite loci. The complementary sequences to two neighboring microsatellites are used as PCR primers so that the variable region between them gets amplified. In order to prevent excessive replication of long DNA sequences, there is a limit to the amplification cycles during this type of PCR. This is useful because the resulting DNA strands will be generally short but varying in length. One must pay attention to the fact that this technique is not capable of distinguishing individuals because an ISSR can be a conserved or non-conserved region.

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DNA barcoding: DNA barcoding is a method of taxonomy and it uses a short genetic marker in an organism and his DNA in order to identify it and then categorize it as belonging to a specific species (42). It is different that phylogeny because here, the main goal is to identify an unknown sample in terms of a preexisting classification (43). Although barcodes are sometimes used in an effort to identify unknown species, (44) the utility of DNA barcoding for these purposes is subject to debate (45). The most famous commonly used barcoding region for animals is a fragment of approximately 600 bp and it belongs to the mitochondrial gene cytochrome oxidase I (COI). This is different in fungi, where part of Internal Transcribed Spacer 2 (ITS2) between rRNA genes is used, and again in plants, where multiple regions are used.

Applications of DNA barcoding include, identifying plant leaves even when flowers or fruit are not available, identifying insect larvae, identifying the diet of an animal based on its stomach contents or feces (46) and identifying products in commerce (for example, herbal supplements, wood, or skins and other animal parts) (43). Having a well characterized and standardized locus for DNA barcoding is optimal. It should also be present in the taxa of interest, easily sequenceable (47), short enough (48) and provide large interspecific than intraspecific variation (49). For plants, it is the concatenation of the rbcL and matK chloroplast genes (47, 50). These provide poor resolution for land plants (51, 52) and a call was made for regions to be assessed that could complement rbcL and matK (52). For fungi, the internal transcribed spacer (ITS) region (53). Kress et al. (43) suggest that the use of the COI sequence "is not appropriate for most species of plants because of a much slower rate of cytochrome c oxidase I gene evolution in higher plants than in animals". A series of experiments was then conducted to find a more suitable region of the genome for use in the DNA barcoding of flowering plants (or the larger group of land plants) (48). One 2005 proposal was the nuclear internal transcribed spacer region and the plastid trnH-psbA intergenic spacer; (43) other researchers advocated other regions such as matK (48).

In 2009, it was proposed by a large group of DNA barcode researchers that the following two chloroplast genes (rbcL and matK) should be used as a barcode for plants (47). The addition of the ITS2 region happened in order to provide a better interspecific resolution (54). The search for the best DNA barcodes regarding plants continues with the latest proposal that the ycf1 region belonging in the chloroplast may be suitable (51).

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55. ISO 9000:2005, Clause 3.2.11

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56. "Quality Assurance vs Quality Control - Learning Resources

57. The Marketing Accountability Standards Board (MASB) endorses this definition as part of its ongoing Common Language in Marketing Project

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