DNA**BARCODING.**

Pioneering Complete Authenticity *at Indena*.

indena®
SCIENCE IS OUR NATURE

OVERVIEW

Work on DNA-based authentication of herbals and botanicals is ongoing. In fact, **while we know a great deal about animals and their DNA, the same cannot be said for plants.**

Our knowledge about animals and, in particular, about their DNA is relatively deep, while authentication of herbs and botanicals is more complex and requires the expertise of different scientific areas: botany, biology and chemistry.

As a matter of fact for plants **there is no universal DNA barcoding methodology** and the choice of a particular technique is often a compromise that depends on a number of factors. **Each plant needs a dedicated method, developed on its own genome.**

DNA sequencing-based tests are emerging as highly reliable and powerful tools to authenticate botanicals, to identify the species and varieties of medicinal plants. They can even be used to identify new species and to create herbal products. They also play **an important role inside the dietary supplement industry, since adulteration is a serious concern**.

Although DNA-based identification technologies are contributing decisively to the authentication of botanicals, they are not the reference methods: **they complement the botanical, chemotaxonomic and metabolomic analytical methods well**. Accordingly, they have to be part of a complete quality texting toolbox, which constitutes a reliable authentication platform.

Throughout its history of almost one hundred years, **Indena has been mapping the genetic identity of the raw materials used for its botanical extracts**. As no universal Genomic ID method is available, **this knowledge has allowed Indena to develop species-specific DNA identification analytical tools.**

As part of its Quality System, Indena is able to apply the appropriate technology to each case.

Sophisticated DNA sequencing-based tests are powerful technologies but they are just one piece of the puzzle: what really counts is gaining knowledge in plant genetic diversity.
This knowledge is part of Indena's very own DNA.

1 THE TREASURE OF NATURE

An instruction manual for life itself: **this is DNA, a molecule that carries the genetic instructions used in all known living organisms.** Despite its complexity, DNA is extremely simple: it is made up of two sugar-phosphate backbones where four molecules, the nucleotides, are linked: cytosine (C), guanine (G), adenine (A) and thymine (T). **Within the different combinations of these four nucleotides and their organization in chromosomes lies biodiversity: and herein can be found the difference between man and an insect, a palm tree and seaweed.**

Plants are marvelously variable, not only in their outer appearance, but also in their inner composition. They are **incredible sources of substances difficult to chemically synthetize**, due to their chemical complexity; they can be classified as primary metabolites and secondary metabolites. Primary metabolites govern all basic physiological processes, which allow plants to grow and seed, while **secondary metabolites are specialized compounds essential for communicating with other organisms in mutualistic or antagonistic interactions.** Here lie the active principles used in producing botanical extracts. An example? Blueberry contains fifteen anthocyanins!

In **1953** the scientists **Francis Crick** and **James Watson** published an article in *Nature* entitled *A Structure for Deoxyribose Nucleic Acid*, a paper that describes the double helical structure of DNA.

Probably their most meaningful phrase was:

"...we have discovered the secret of life!"

Plant genetics are different from that of animals and the gene pool is even more complex.
In plant cells there are three different genomes: nuclearus, mitochondral and chloroplast. Additionally, unlike humans with their two sets of chromosomes, a large set of spontaneous and cultivated plants have more than two paired (homologous) sets of chromosomes, they are called polyploids; for example, potatoes, peanuts, and cotton have four sets of chromosomes, sugarcane, oats and bread wheat have six sets of chromosomes.

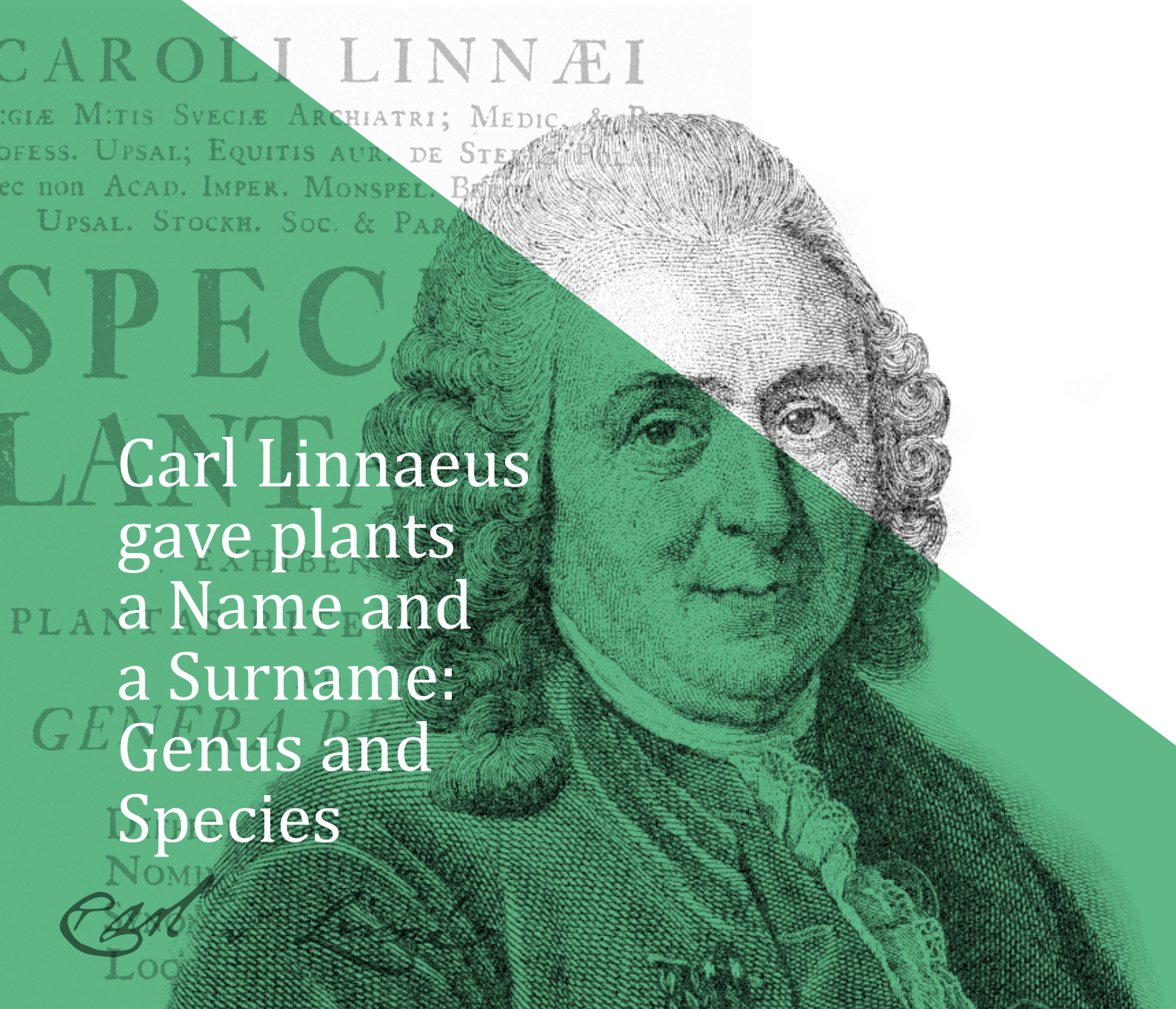
PLANT IDENTIFICATION: HOW TO MANAGE THE CLASSIFICATION OF PLANTS?

Sometimes it is difficult to identify a plant through a morphologic exam. Working with plants requires specific skills to handle their complexity and, consequently, to know exactly their secondary metabolite pattern. **The quality of herbal preparations relies strictly on the origins and quality of the initial herbal material.**

Plant taxonomy is the science used to identify and classify plants.

The set of rules for formal nomenclature is governed by the **ICN (International Code of Nomenclature** for algae, fungi and plants). The origin of these techniques dates back to **Carl Linnaeus**, a Swedish botanist, physician and zoologist who **formalized the modern system of naming organisms called binomial nomenclature**. It is a formal system of naming living beings with an appellation consisting of two parts: **the first part identifies the genus to which the species belongs; the second identifies the species within the genus.**

Plants of the **same genus, however, may have different species with different gene pools**, expressing different secondary metabolites. Moreover, the species and varieties being equal, the expression of **genetic identity of a plant is influenced also by the environment** where the plant lives and grows: individuals of the same species that grow in different places could well have a **bouquet of different secondary metabolites.**



Carl Linnaeus gave plants a Name and a Surname: Genus and Species

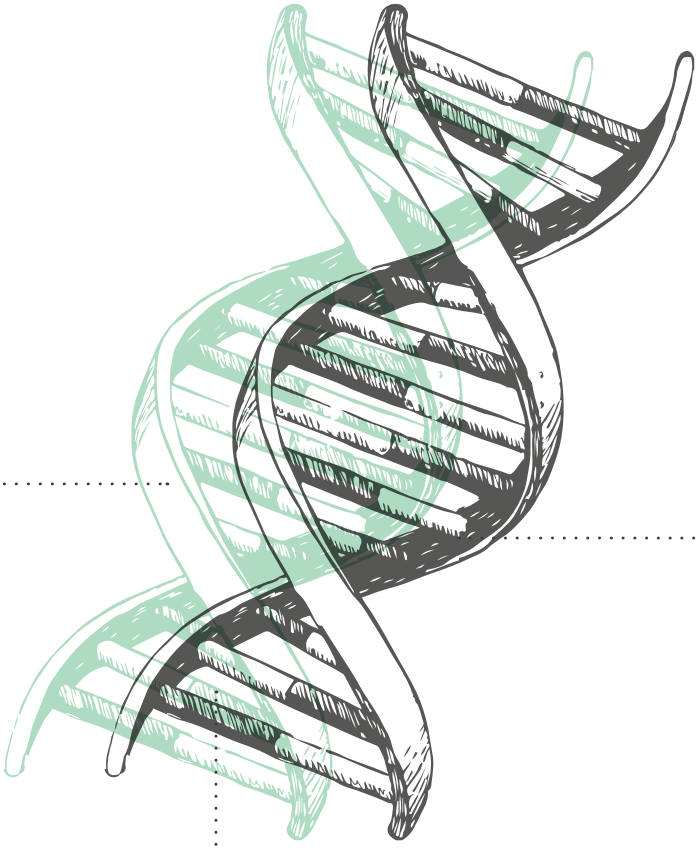
2 WHAT IS DNA TESTING?

The first step in quality control of botanical preparations is **ensuring the correct identification of the plants used**. So far, there are two mandatory analytical tests: **botanical identification** (macroscopic and microscopic) and **chemical identification** (fingerprint analytical techniques for the characterization of secondary metabolites).

The two main references are the **European Pharmacopoeia** – General Chapter Herbal – and the **United States Pharmacopoeia** – General Chapter Identification of Article of Botanicals Origin, accepted all around the world as the main references.

Most plants can be unequivocally identified by a macroscopic and microscopic analysis. **In some cases however, it is necessary to resort to DNA-based methodologies to precisely define the identity at the species or variety levels.**

There are three stages of DNA testing:



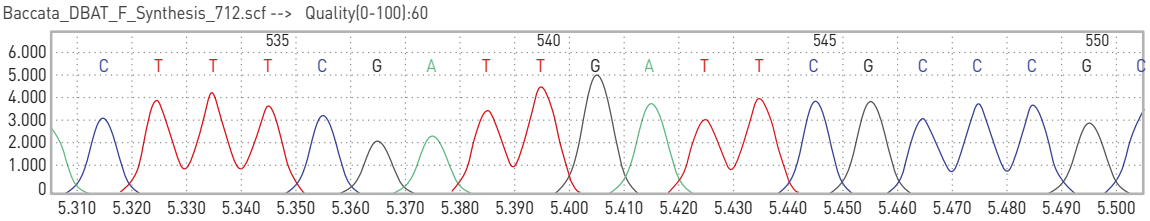
1
EXTRACTION
through a standard protocol to extract the DNA

2
AMPLIFICATION
usually made through PCR – Polymerase Chain Reaction, in order to have a consistent amount of specific DNA to be analyzed

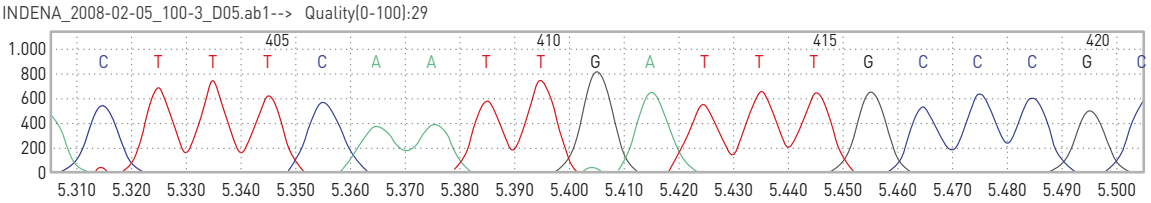
3
MEASUREMENT
by analysis of DNA sequences

Differences of the nucleotides distribution in the target DNA sequence for two species of Taxus

Taxus Baccata



Taxus X Media



DNA barcoding can be useful in assigning a dry extract to its species of origin.

Differences of the nucleotides distribution in the target DNA sequences for different species of Taxus
11 single-nucleotide polymorphism (SNP) that occur at a specific position in the genome are needed to differentiate the target species

	SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	SNP 6	SNP 7	SNP 8	SNP 9	SNP 10	SNP 11
T. BACCATA	T	G	C	CT	G	GA	C	A	T	A	G
T. W. WALLICHIANA 1	T	G	C	C	G	G	C	A	T	A	G
T. W. WALLICHIANA 2	T	G	C	C	G	G	C	AC	T	A	G
T. FUANA	T	G	C	CT	G	A	C	A	C	A	G
T. BREVIFOLIA	T	G	C	T	G	G	C	C	T	GA	G
T. GLOBOSA	T	G	C	T	G	G	C	C	T	A	A
T. M. WARDI	T	G	CT	CT	GC	A	C	AC	T	A	G
T. M. BROWNI 1	T	G	CA	T	G	GA	C	AC	T	A	G
T. M. BROWNI 2	T	G	CA	T	G	GA	CG	AC	T	A	G
T. CANADENSIS	T	G	C	T	G	G	G	C	T	A	G
T. M. TAUTONI	T	G	C	T	G	GA	G	C	T	A	G
T. M. DGS	T	G	T	T	G	A	C	C	T	A	G
T. CUSPIDATA	T	G	CT	T	G	A	C	C	T	A	G
T. M. DENSIFORMIS	T	G	CT	CT	G	GA	C	AC	T	A	G
T. FLORIDANA	T	G	C	T	GC	G	C	C	T	A	G
T. M. RUNYAN	T	G	CT	T	G	GA	C	AC	T	A	G
T. M. HICKSII	T	G	CT	T	G	A	C	AC	T	A	G
T. W. MAIREI 1	CT	GT	T	T	G	G	C	A	T	A	G
T. W. MAIREI 2	CT	GT	T	T	G	G	C	C	T	A	G
T. W. CHINENSIS	CT	G	CT	CT	G	G	C	C	T	A	G

The main difficulty in applying DNA identification techniques in the plant kingdom is that **there is no universal plant DNA barcode useful for identificative purpose** unlike in animals, where a segment of the mitochondrial gene cytochrome oxidase I (COI) is commonly used. DNA identification in plants on the other hand would require investigation of several genes (e.g. ITS1, ITS2, rbcL, matK trnH-psbA, trnL-trnF, etc.).

Additionally, unlike with animals, the main pitfall of botanical DNA identification is that **there is no check for origin in the DNA genomic data deposited in the Genbank public Database**. This could give rise to potential attribution **errors of DNA sequences** to the correct species or variety.

Successful development of DNA-based authentication tools for plants and botanicals of unknown origin would require the creation of **"reference" DNA profiles** which would be shared and validated by the whole scientific community. These "reference" DNA profiles should derive **from the analysis of plant samples of known, certified origin (herbarium, taxonomically identified samples).**

Based on the above assumptions it is of paramount importance to have "certified" pure samples in order to derive reference DNA sequences for use in analyzing DNA data.

As yet, the botanical extracts industry does not have any such assurance.

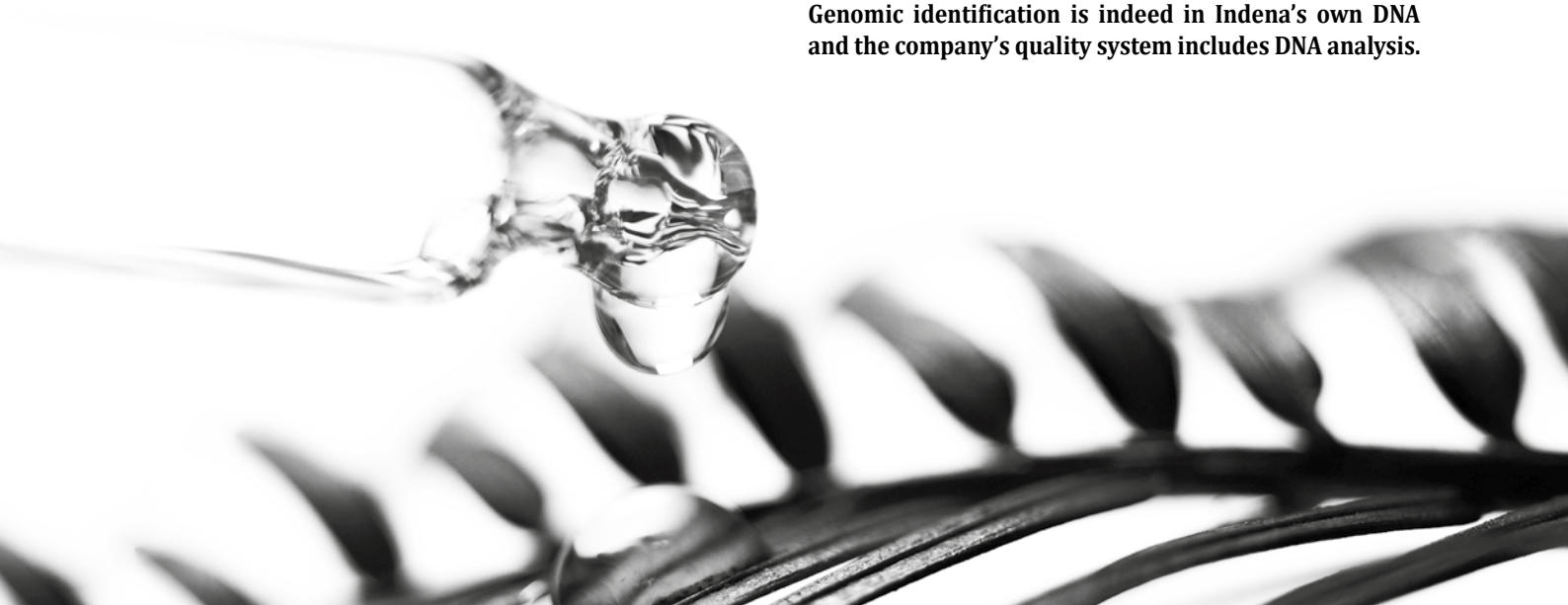
Indena is able to recover samples to authenticate the identification of plants on a scientific basis, through botanical analysis.
This unique and extensive broad knowledge enables the company to go even further.

3 INDENA AND DNA TESTING: GENOMIC ID IS IN OUR DNA

Quality is one of the pillars of Indena. We strongly believe that in-depth knowledge of medicinal plants and the search for excellence are crucial commitments to serving the customers in the pharmaceutical, health-food and personal care industries. **Our products for the pharmaceutical market meet the US-FDA and EU requirements**, and the strict quality controls we apply to all production goes well beyond the mandatory quality analyses, whether botanical, taxonomical or chemical.

All five Indena plants are cGMPs compliant and are authorized for the production of APIs. Four plants work under the HACCP and are authorized for the production of food ingredients. We are routinely inspected by the FDA, AIFA, and ANSM for GMP compliance assessment as well as by regulatory authorities for food safety compliance.

Genomic identification is indeed in Indena's own DNA and the company's quality system includes DNA analysis.



Indena's approach to genomic identification can be summarized as follows:

Indena DNA barcoding is *orthogonal*, because one single technique may not be enough.

Indena DNA testing is *affordable* because we can use the whole range of available techniques, from DNA barcoding to DNA-NGS.

Indena draws on its ***botanical background*** to structure the DNA-based methodologies.

Indena knows ***where, when and how to collect*** the reference species to be used as "certified" controls to validate DNA-based methods.

The company's expert botanists are also able to identify ***unknown species***.

Indena has the *availability of authenticated plant material* to develop highly reliable DNA-based tests. This is its heritage.

Indena started integrating DNA sequencing into its Quality System in 2011, more recently taking advantage of **modern NGS technologies where required**: a precise database for starting material has been put together since then.

Most of the best-selling Indena products are already DNA tested, using the most appropriate technique.

Some examples of the most important target genera:

Actaea
Curcuma
Cynara
Echinacea
Eleutherococcus
Equisetum
Euphorbia
Ginkgo
Harpagophytum
Illicium
Kalanchoe
Panax
Silybum
Taxus
Vaccinium
Vitis

4 ON-SITE ANALYSIS: THE FUTURE IS NOW

Since **building a solid genomic database is essential,** to produce species-specific methods which can distinguish one particular plant from another and thus from adulterants, **we are always eager to harness the best available means of achieving this.**

Next Generation Sequencing (NGS) is the term given to the most refined techniques known today. DNA mapping is carried out using **advanced methods and various technologies**, but in all cases the output is very precise and reliable, **even when materials are complex and contain very low quantities of DNA.**

NGS technologies are based upon high-throughput decoding of all DNA present in a given extract. They handle millions of small DNA fragments with an untargeted approach that generates valuable data to assess the presence of adulterants and assign all product ingredients at the species level.

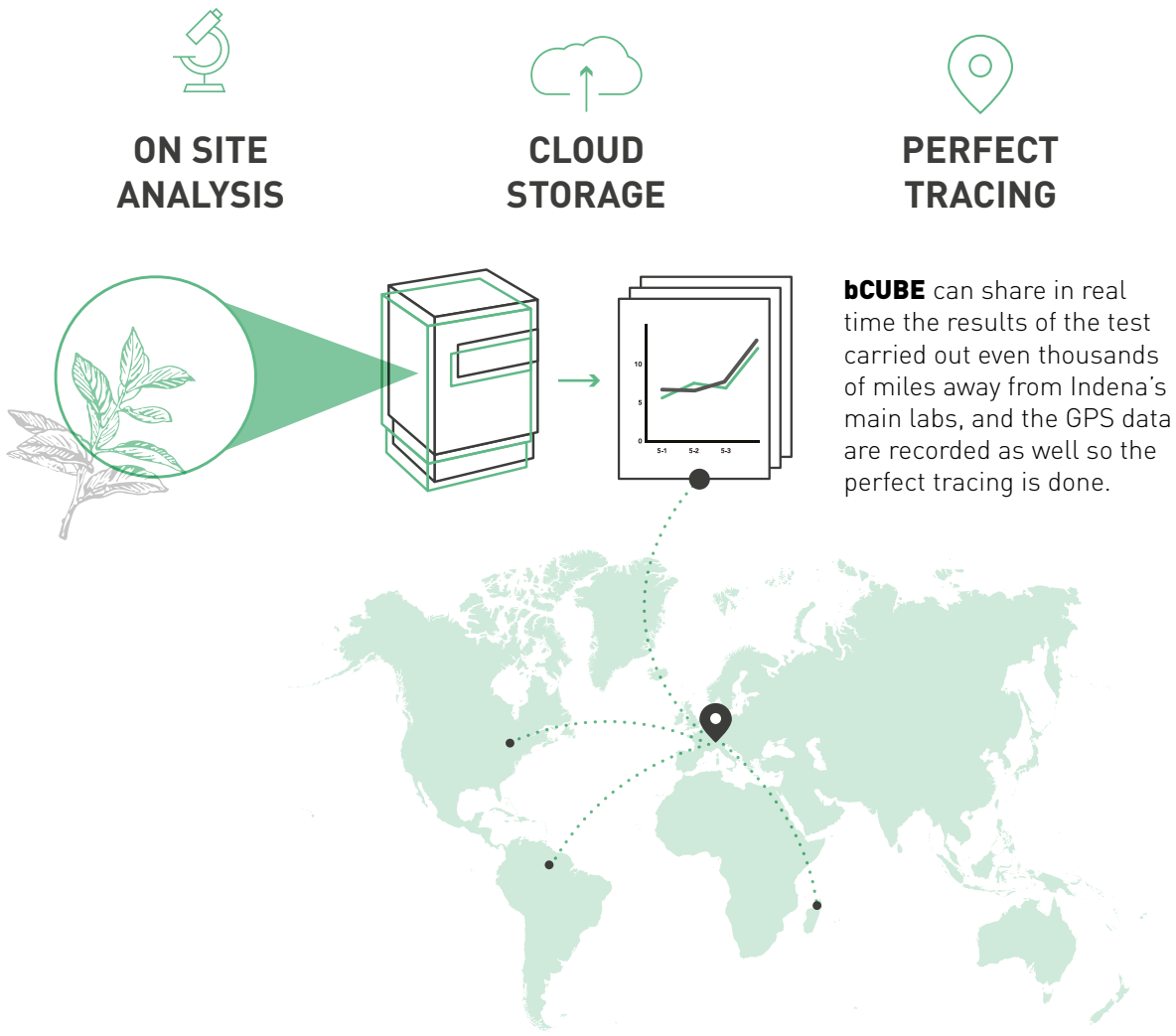
Indena is working to validate proper DNA-based technologies to also include these tests on the final extracts.

Indena has gone much further, however. Through an exclusive agreement with Hyris Ltd, and drawing on the solid Indena database of reference compiled over decades, **DNA fingerprint analysis on plant samples can be carried out in the field even by unskilled personnel.**

The device that makes this is the bCUBE, produced by Hyris.

As small as a coffee mug, the bCUBE can analyze starting material straight from the field in roughly an hour. **Even when carried out thousands of miles away from the Indena labs, these test results can then be shared on a proprietary cloud-based platform in real time, and the GPS data recorded to enable perfect tracing.**

bCUBE: fast processing and wireless connectivity



bCUBE can share in real time the results of the test carried out even thousands of miles away from Indena's main labs, and the GPS data are recorded as well so the perfect tracing is done.

Indena holds preferential access to this breakthrough genomic technology for on-site identification of botanical species, exclusively for the dietary supplement ingredient industry.

This is another pioneering high added value solution **Indena is able to offer partners seeking excellence in botanical extracts.** In this way, the dietary supplement market at last becomes a place where both producers and consumers can find clear answers regarding the authenticity of the products they sell or buy.

MULTI-INGREDIENT PRODUCTS

The NGS approach is the only analytical means enabling the precise identification of the composition of multi-ingredient products. Their precision and applicability rely on the presence of DNA even if only in traces and on the use of four universal barcodes representing both chloroplast and nuclear genomes. The use of multiple barcodes ensures that the identification of the ingredients, fillers and adulterants is always achievable at the species level.

NGS methods also provide semi-quantitative data of the ratios of the DNA assigned to a specific species in multi-ingredient or adulterated samples. The only limitation on the use of NGS analytical tools is the complete degradation of DNA caused to botanicals by harsh treatments in manufacturing procedures such as supercritical CO_2 and the solvent extraction process.

5 GOING FURTHER – HOW INDENA MAKES THE DIFFERENCE

In recent years, the issue of potentially mislabelled botanical products has raised questions about product quality and safety, and has led to concerns in general consumer confidence, especially in dietary supplements.

At Indena we have always followed a rigorous scientific approach in our research and we have leveraged this approach as well as our solid pharmaceutical background in the health-food market, where we develop biologically active ingredients for supplements, functional, medical and baby food products. In our long history, **we have developed a huge number of products with a distinctive high quality profile,** starting from a state-of-the-art clinical study design with the aim of meeting and even exceeding market expectations, turning challenges into opportunities. **All the best products Indena brings to the market have undergone a standardization process and their activity in terms of efficacy and safety is scientifically documented.**

We know that the use of every species of plant and sometimes specific varieties within one species may have substantially different consequences on the results obtained at the end of the production process, in terms of active ingredients. **In this scenario, it is necessary to have a reliable genomic analysis, managed by experts who know and understand the plant kingdom and how to evaluate the best techniques in order to obtain the most useful information.**

Our approach in DNA barcoding is aimed at facing this challenge properly. Indena expertise in DNA barcoding puts together an impressive array of powerful DNA-based technologies, including DNA fingerprinting, PCR-based tools, specific DNA-Next Generation Sequencing.

No universal Genomic ID is available: for each plant genus, the method must be developed and validated with proper specimens.

Indena's approach in DNA barcoding is completely reliable:

- The experienced botanists amongst our researchers are able to identify even species as yet unknown.
- We are able to use all techniques.
- We have developed the methods.
- We know how to explore plant varieties.

INDENA DNA TESTED

As a guarantee to partners, a special Indena logo has been created to demonstrate that the starting plant material used for the manufacture of the extract has also been DNA tested. In addition to the Indena quality system, our clients can count on the reliability of the database developed over 90 years by our committed botanists and their deep-seated understanding of nature.

DPR0268 - 07/2017