

**ESA WHITE PAPER
ON
PLANT METABARCODING NEXT GENERATION SEQUENCING (NGS) ANALYSIS
APPLIED TO CULINARY HERBS AND SPICES**

(Version 2 - June 2021)

MAIN POINTS

- **NGS is not able to detect any adulterations with economically motivated additions of endogenous material.**
- **NGS can be a secondary tool to confirm and identify only additions of exogenous plant matters containing DNA when detected by reference methods.**
- **NGS is not able to detect any adulterations with economically motivated additions of materials not containing DNA.**
- **NGS should not be used as a direct tool to authenticate herbs and spices nor to detect or quantify them as ingredients in mixtures.**
- **Reference methods like classical microscopy or validated non-targeted chemical/physical methods (using NMR, NIR/MIR spectroscopy, mass spectroscopy,) or a combination of them should be used as primary analyses to prove herb and spice authenticity.**
- **NGS methods using short DNA fragments should be chosen to mitigate the effect on analysis results of processes applied to herbs and spices (milling, steam-treatment, etc.). Any internal herb and spice databases should be stated, and the use of reliable reference materials should be demonstrated.**

TECHNICAL BACKGROUND

1) Endogenous materials

A spice is a well-defined part of the plant it belongs to. It can be either the fruit, the bark, the stigma of the flower, the bud, the seed, the root, the rhizome, the leaf, the kernel or the bulb. And any economically motivated addition of other parts of the same plant is considered as a fraud.

NGS is not capable of distinguishing between parts of the same plant so unable to authenticate spices versus fraudulent endogenous matter additions.

Example

100% of ground pepper leaves or 100% of ground black peppercorn or a mixture of them will all be identified as 100% pure *Piper nigrum*.

For that reason, only the plant name (Latin and vernacular names) should be reported on NGS plant certificates of analysis not the spice name.

2) Exogenous materials

Component percentages given by NGS analysis are DNA sequence read percentages which tightly depend on:

- the quantity of DNA the plant contains;
- the DNA recovery rate of the extraction step for the considered plant.

It occurs that spices are difficult matrices sometimes containing low levels of DNA what is more, difficult to extract (cinnamon/cassia, black pepper, etc.). Consequently, the ratio of other natural foreign plant matters, if any, can be increased and thus artificially overestimated.

The DNA amount does not correlate with the weight amount of a plant in a mixture and thus the DNA sequence read percentages reported do not give the weight percentages. Such distorted results may mislead the customer.

Consequently, NGS quantitation of herbs and spices may create unfortunate confusion between adulteration and natural presence of foreign matters.

3) Other materials

In herbs and spices **NGS methods are not able to detect fraudulent additions of exogenous materials that contain no DNA** or highly fragmented DNA.

Example

Artificial chemical dyes, extracted/defatted materials, minerals (salt, talc, sand, chalk, brick, etc.), highly processed materials (starch, maltodextrin, sugars, oil, etc.), etc.

4) Composition of mixtures containing herbs and spices

NGS cannot be used to verify the herb and spice composition of a mixture of them as ingredients for the following reasons:

- As described in point 2), the DNA read percentages are misleading because they cannot be related to actual weight percentages especially for spices. **So, there is no possibility to check the real weight percentages of an ingredient list.**
- As described in point 2), some spice ingredients can be missed because of **their low DNA level and the difficulty to be extracted.** Extraction procedures also vary between labs, **there is still no norm on the subject for standardised extraction.**
- **Some spice ingredients can be missed** because of the process they have been subjected to (process such as grinding/milling, colouring, bleaching, fumigating, steam treatment or drying may have an influence on the DNA quality jeopardising successful analyses). The shorter the DNA fragments used are, the less sensitive to process the method is.

5) Reference methods

Reference methods like classical microscopy or validated non-targeted chemical/physical methods (using NMR, NIR/MIR spectroscopy, mass spectroscopy,) **or a combination of them should be used as primary analyses** to prove herb and spice authenticity.

Please see this link to the Food Integrity Handbook:

<https://secure.fera.defra.gov.uk/foodintegrity/index.cfm?sectionid=83>

In any case, **NGS methods using short DNA fragments should be chosen to mitigate the effect on analysis results of processes applied to herbs and spices** (milling, steam-treatment, etc.).

The **plant DNA databases** used (NCBI, internal databases, ...) and their version should be stated. The list of the genera/species they contain along with the origin of the entries should be available to the end-users.

PRACTICAL BACKGROUND

REPORT OF A PLANT NGS INTERLAB TRIAL ORGANISED BY ESA IN 2020

Goal

To assess commercial laboratories upon their competence to detect plant adulterants in herbs and spices at economically motivated adulteration levels.

Time frame

- February 2020: launching of the project.
- March-July 2020: selection of the NGS laboratories, spiked samples preparation by volunteer companies.
- August-October 2020: contacts with the NGS laboratories, sample shipments and result collection.
- November 2020: NGS result analysis, laboratory scoring.

Samples

Samples were genuine herbs or spices spiked with multiple plant adulterants. These adulterants were chosen among the most relevant and common ones for each herb and spice.

One culinary aromatic herb and 5 spices were selected as well as 22 adulterants. The latter were added at levels ranging from 5 to 20% w/w (see table 1) in one single spiked sample per herb/spice. A foreign plant was chosen (bindweed) as a natural agricultural contaminant and added at a 1% w/w level in oregano.

Each spiked sample was thoroughly ground. 100-150g samples were shipped to each lab except for saffron for which only a 10g samples were sent.

Laboratories

Six laboratories, five of which based in Europe, were selected among the most used ones for the plant NGS testing and applied to herb and spice identification/authentication.

Five of them were ISO17025 accredited for plant NGS testing. They used either Ion Torrent or Illumina technology for sequencing (see table 2). Two of them provided quantitative results expressed in DNA reads percentages.

Instructions to laboratoires

Samples were labelled according to the vernacular English name of the herb/spice they were supposed to contain. Laboratories were required to analyze the samples using their regular QC plant NGS protocol.

Products <i>Plant Latine name</i>	Origin	Note	Adulterants	% adulterant	Total % adulterant
Oregano <i>Oreganum vulgare</i> 50% <i>Oreganum onites</i> 50%	Turkey	Oregano and adulterants not sterilised	olive leaf	10%	56%
			myrtle leaf	10%	
			cistus leaf	10%	
			sumac leaf	10%	
			hazel leaf	10%	
			thyme	5%	
			<i>Convovulus</i> (bindweed)	1%	
Turmeric <i>Curcuma longa</i>	India	Turmeric and adulterants not sterilised, Boiled turmeric	tapioca flour	10%	30%
			rice flour	10%	
			corn flour	10%	
Black pepper <i>Piper nigrum</i>	Vietnam	heat-treated pepper, not-sterilised adulterants	coffee husk	20%	40%
			sawdust	20%	
Cumin <i>Cuminum cyminum</i>	India	heat-treated cumin, not-sterilised adulterants	peanut shell	10%	40%
			rice starch	10%	
			almond kernel	10%	
			grass seed (<i>Poa annua</i>)	10%	
Saffron <i>Crocus sativus</i>	Iran	no sterilisation, boiled turmeric	safflower	10%	40%
			turmeric	10%	
			paprika	10%	
			dry beetroot	10%	
Paprika <i>Capsicum annum</i>	different origins	Heat-treated paprika, not-sterilised adulterants	almond shell	15%	45%
			tomato skin	15%	
			wheat starch	15%	

Table 1

Laboratory scoring protocol

The laboratory score calculation took into consideration:

- whether the right herb/spice is detected,
- whether the herb/spice was detected down to the species level (genus or family level is not acceptable as most of spices come from one specific plant species),
- the number of detected adulterants,
- whether the adulterants are detected down to the species (perfect), genus (acceptable) or family (not acceptable).

A lab fulfilling all these criteria gets a maximum score of 35.5 as presented in the raw table of results (Table 5 in appendix). For the sake of simplicity, this 35.5 maximum score is converted into 10 in the final results (Table 2).

Interlab trial results

Lab number	1	2	3	4	5	6
ISO17025 accreditation	Y	Y	Y	Y	N	Y
Quantitative results	Y	N	N	Y	N	N
Sequencing technology	Illumina	Ion Torrent	Ion Torrent	Illumina	Illumina	Illumina
LoD (in DNA reads %)	0,2%	0,5%	1,0%	0,5%	0,5%	0,5%
FINAL SCORE (max score=10)	7,7	5,4	5,6	1,8	2,8	6,2

Table 2

(more details in table 5 in Appendix).

COMMENTS ON RESULTS

General observations

- Some laboratories reported quantitative results (expressed on DNA reads percentage, see Table 2), others classified the results in the descending order. Absence of a standardised way of delivering results.
- Most of the laboratories displayed a note in their certificate of analysis mentioning that the DNA reads percentages do not reflect the weight percentages for the ingredients.
- Inappropriate ways of presenting results have occurred that can be confusing for the customer:
 - ✓ peppercorn (*Piper nigrum*)
 - ✓ chilli or Cayenne pepper (*Capsicum* spp.)
 - ✓ paprika or chilli pepper (*Capsicum annum*)

Other comments

Black pepper results proved the DNA read percentages do not reflect the weight percentages (see table 3). The quantity of black pepper is clearly underestimated using NGS.

	Sample mass percentage	DNA percent (Lab1)	DNA percent (Lab 4)
Black pepper	60 %	3,4 %	7,6 %
Coffee husk	20 %	70,3 %	92,4 %
Sawdust	20 %	-	-

Table 3

- Ten adulterants (45%) were missed or detected by very few laboratories:
 - ✓ olive and sumac leaf in oregano,
 - ✓ tapioca/corn flour in turmeric,
 - ✓ turmeric and beetroot in saffron (turmeric is traditionally boiled before being dehydrated),
 - ✓ Almond and peanut shell, rice and wheat starch (low level of DNA or highly degraded DNA).

- Sawdust is a difficult adulterant to be detected by NGS, because its composed of many tree species. Also, the detection of the tree species does not allow the identification of whether it is bark, leaf, fruit, root, etc.
- Unexpected plants were detected :
 - ✓ *Amaranth* sp. detected in oregano and cumin spiked samples (weeds from *Amaranth* genus are known to be resistant to herbicides),
 - ✓ corn DNA in paprika,
 - ✓ fenugreek DNA in saffron,
 - ✓ wheat DNA in black pepper.

At this stage, it cannot be said whether the unexpected plants came from the herbs/spices or from the adulterants.

- Bindweed was added as a natural agricultural contaminant (1%) in oregano and its quantity is clearly overestimated using NGS quantitation (see table 4).

	Mass percentage in sample	DNA percentage (Lab 1)	DNA percentage (Lab 4)
Bindweed	1,0 %	4,4 %	9,7 %

Table 4

ESA recommendations to laboratories using plant NGS to authenticate culinary aromatic herbs and spices:

Because DNA is common to any parts of a same plant and a spice is a part of a plant, plant NGS certificates of analysis should not mention any spice names but only the Latin name of the detected plant. When a species name is not obtained, the result can be displayed using other taxonomic levels (e.g genus, family, etc).

APPENDIX: NGS interlab trial, raw table of results.

	Lab 1		Lab 2		Lab 3		Lab 4		Lab 5		Lab 6		Notes	
	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y		
ISO17025 accreditation														
Sequencing technology	Illumina													
LoD	0,2%													
OREGANO	Nb of detected spices (2)	2	1	2	1	1	1	1	0	0	2	2	- None found olive and sumac leaf - Bindwind was overestimated - Unexpected <i>Amaranth</i> sp. was detected by 3 labs	
	Taxonomic level of the detected spices	species	species	species	species	species	genus	genus	-	species	species	species		
	Nb of detected adulterants (7)	5	4	2	5	3	3	3	3	3	4	4		
	Taxonomic level of the detected adulterants	species	sp./genus	species	sp./genus	gen./family	sp./genus	sp./genus	gen./family	gen./family	sp./genus	sp./genus	sp./genus	
	Number of other plant detected	3	1	0	1	1	1	1	7	3	3	3		
TURMERIC	Nb of detected spices (1)	1	1	1	1	1	1	1	1	1	1	1	- None found tapioca and corn flours - Lab 1 detected unexpected oregano (lab contamination)	
	Taxonomic level of the detected spices	species	species	species	species	species	genus	genus	species	species	species	species		
	Nb of detected adulterants (3)	1	1	1	1	1	1	1	1	1	1	1		
	Taxonomic level of the detected adulterants	species	genus	genus	family	genus	family	family	clade	clade	species	species	species	
	Number of other plant detected	6	0	1	1	1	1	1	5	2	2	2		
BLACK PEPPER	Nb of detected spices (1)	1	1	1	1	1	1	1	1	1	1	1	- Pepper DNA read percentage far below that of coffee - Unexpected wheat was detected by 4 labs	
	Taxonomic level of the detected spices	species	species	species	species	species	genus	genus	species	species	species	species		
	Nb of detected adulterants (2)	2	2	2	1	2	1	1	2	2	2	2		
	Taxonomic level of the detected adulterants	species	genus	sp./gen./fam.	genus	family	genus	genus	family	family	genus	genus	genus	
	Number of other plant detected	3	1	1	0	1	0	0	0	0	1	1	1	
CUMIN	Nb of detected spices (1)	1	1	1	1	1	1	1	1	1	1	1	- Peanut shell only detected by 2 labs, none detected rice starch - Unexpected <i>Amaranth</i> sp. was detected by 3 labs	
	Taxonomic level of the detected spices	species	species	species	species	species	genus	genus	species	species	species	species		
	Nb of detected adulterants (4)	2	4	1	1	3	1	1	3	2	2	2		
	Taxonomic level of the adulterants	species	genus	genus	genus	gen./family	genus	genus	gen./family	gen./family	genus	genus	genus	
	Number of other plant detected	2	2	0	2	0	2	2	0	0	1	1	1	
SAFFRON	Nb of detected spices (1)	1	1	1	1	1	1	1	1	1	1	1	- Unexpected fenugreek detected by 4 labs - Only one found turmeric. None found beetroot	
	Taxonomic level of the detected spices	species	species	species	species	species	genus	genus	species	species	species	species		
	Nb of detected adulterants (4)	2	2	2	2	2	2	2	2	2	2	2		
	Taxonomic level of the detected adulterants	species	genus	species	species	species	sp./genus	sp./genus	gen./family	gen./family	species	species	species	
	Number of other plant detected	6	0	1	1	1	1	1	4	4	1	1	1	
PAPRIKA	Nb of detected spices (1)	1	1	1	1	1	1	1	1	1	1	1	- Unexpected corn detected by 4 labs	
	Taxonomic level of the detected spices	species	genus	species	species	species	species	species	species	species	species	species		
	Nb of detected adulterants (3)	3	1	2	1	1	1	1	1	1	1	1		
	Taxonomic level of the detected adulterants	species	genus	sp./genus	genus	family	genus	genus	family	family	species	species	species	
	Nb of other plant detected	3	1	1	1	2	1	1	2	2	1	1	1	
Results	perfect	20	13	15	7	11	16	16	16	16	16	16	lab score = nb green boxes + 0.5 x nb of detected adulterant - nb of red boxes	
	incomplete	7,5	7	5	5,5	6	6	6	6	6	6	6		
	non-acceptable	0	1	0	6	7	0	0	0	0	0	0		
	Final score (max=35.5)	27,5	19	20	6,5	10	22	22	22	22	22	22		

Table 5