Differences between the measurement of phenolic ingredients of olive oil by NMR and HPLC-UV

as described in the official method of International Olive oil Council (IOC)

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It has been 10 years since the initial publication of the method for measuring the phenolic components of olive oil using the NMR method and until now with this method more than 10,000 samples have been analyzed.

We are often being asked why this method gives more reliable results in substantiating the health claim than the HPLC method proposed by the International Olive Oil Council. In this article we will try to explain in the simplest possible way why the IOC method is inappropriate and why it underestimates olive oils that are rich in oleocanthal in comparison to those that contain oleacein or oleuropein aglycon.

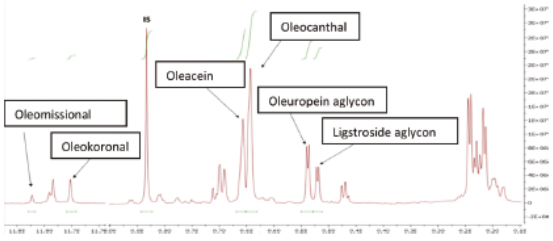
Explanation of the basic principles for measurement of phenolics by each method.

The basic difference is that the NMR measures signals directly proportional to the number of atoms and molecules in a specific amount of sample while the HPLC measures signals related to light absorbance and which are indirectly correlated with the number of molecules in a sample using an arbitrary correlation factor.

•qNMR (quantitative nuclear magnetic resonance spectroscopy)

More specifically in the NMR spectrum we can observe peaks corresponding to specific hydrogen atoms of a specific molecule. The area of the peak is proportional to the number of atoms corresponding to the peak and consequently to the number of molecules corresponding to this peak. Using an internal standard of known amount, we can correlate the known number of molecules of the internal standard with a specific area of a specific peak. Comparing the area of the peak of the internal standard with the peak of the specific phenolic compound that we want to measure we achieve a direct measurement of the amount of the target compound.

The NMR spectrum is recorded in a solution of an inert solvent like CDCl3 which cannot react with any of the measured phenols and in addition there is no static phase, no pumps, no UV lamps, no moving parts (e.g injectors) that could interfere with the analysis. The sample is inserted into the magnet and the measurement is completed in less than 60 sec.



In the above example the area of the peak of the internal standard (IS: syringaldehyde) corresponds to a known number of hydrogen atoms and a specific known amount of the molecules of the internal standard, since each molecule contains one hydrogen atom able to resonate at the specific frequency of the aldehydic hydrogen of syringaldehyde. If we compare the area of the peak of the internal standard with the area of any of the peaks corresponding to specific hydrogen atoms of each specific compound that we want to measure, we can directly measure the number of hydrogen atoms corresponding to each peak and consequently the number of molecules of each compound, since again each atom corresponds to one molecule.

The measurement of the area of each peak (called

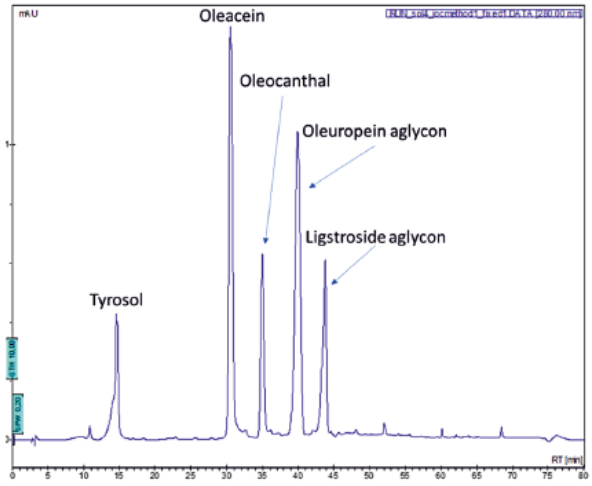
integration) is a mathematic procedure performed automatically by the software of the NMR instrument and is not influenced by the user. The results of the NMR analysis are expressed as mg of each compound per Kg of olive oil

HPLC-UV (high performance liquid chromatography coupled with UV detector)

The method proposed by the IOC for measurement of total biophenols is based on the chromatographic separation of each phenolic compound and the indirect measurement of the amount of each compound using the absorption of the UV light from each compound the moment that the compound is eluted from the separation column and passes through a chamber with a UV lamp. The light absorption is plotted against time and the amount of each compound is proportional to the area of the peak of each compound. The recorded plot is called a chromatogram. It is not a spectrum as in the case of NMR. The area of the peak of each compound is compared with the area of the peak of a known amount of tyrosol and the results are expressed as equivalents of tyrosol in mg per Kg of olive oil.

The errors that occur during this measurement are the following:

The area of the peak of each compound is theoretically proportional to the number of molecules of each compound but the area is also determined by the capability of each molecule to absorb the UV light at a specific wavelength. This means that if two compounds are present in a mixture at equal number of molecules but the first one absorbs the UV light twice more than the second one, then the area of the peak of the first compound will be double in comparison with the second compound. So if the two peaks are compared with the known area of the peak of tyrosol then the first compound will correspond to double equivalents of tyrosol in comparison to the second one although they are both present at equal number of molecules.



As can be seen from the above HPLC chromatogram in which equimolar quantities of tyrosol, oleocanthal, oleacein, oleuropein aglycon and ligstroside aglycon have been tested, it is obvious that they give a completely different area and therefore concentration in olive oil. This is a very critical mistake because all the phenolic ingredients of olive oil that contain the moiety of hydroxytyrosol (like oleacein, oleuropein aglycon and oleomissional) have almost double capability to absorb UV light in comparison with the molecules that contain the tyrosol moiety (oleocanthal, ligstroside aglycon and oleokoronal). In this sense olive oils that contain more hydroxytyrosol derivatives than tyrosol derivatives appear artificially to be richer in total phenolics when the results are expressed as tyrosol equivalents.

The above type of mistake overestimates the phenolic content of olive oils rich in oleuropein aglycon and oleacein (e.g. Picual, Coratina) and underestimates the olive oils rich in oleocanthal (e.g. Koroneiki, Kalamata). However, if we compare HPLC results among olive oils of the same variety, the relative comparison is generally correct.

Another problem is that the official HPLC method measures (integrates) the area of all detected peaks at the wavelength of 280 nm and consequently the total phenolic content includes also molecules that are not included in the list of compounds related with the EU health claim (e.g. lignans, phenolic acids, flavonoids etc). In most cases the total phenolic content is not significantly influenced by those compounds because usually they are present in much smaller amounts than the secoiridoids phenolics derivatives of hydroxytyrosol and tyrosol. However, some varieties contain big amounts of lignans and for this reason the HPLC method overestimates their total phenolic content. Moreover, aged hydrolysed olive oils contain big amounts of free tyrosol or hydroxytyrosol which are obligatory measured and included in the total phenolic content result. For this reason the total phenolic content measured by HPLC cannot discriminate the old hydrolysed olive oils from the fresh or well preserved ones.

Another type of mistake is that sensitive compounds like oleocanthal or oleacein interact with solvents of the mobile phase like water or methanol and can be transformed to hydrated or methylated hemiacetals which do not naturally exist in the olive oil. The above problems can be partially overcome using calibration curves constructed with pure oleocanthal or oleacein but until now this is not included in the official method. For the above reasons the HPLC method underestimates dramatically the real oleocanthal and oleacein content.

Concerning compounds like oleuropein aglycon and ligstroside aglycon it should be noted that during chromatography they are getting partially isomerized (from open to closed ring forms) and again the real content of the olive oil for each compound cannot be measured. The closed ring forms are overestimated in comparison to open forms (oleokoronal and oleomissional). Due to all the above reasons the HPLC chromatograms become very complicated because each compound is transformed to several artificial derivatives or isomers and consequently the peaks are heavily overlapped and the exact area of each peak is difficult to be measured. For this reason, the official method should not be used for the measurement of each independent compound but only for the total phenolic content. Although this is mentioned in the protocol of the official method several laboratories use erroneously the HPLC-UV method to measure independent phenolic compounds and express their results as tyrosol equivalents.

Again, the relative comparison among different olive oils of the same variety with the HPLC method is generally correct since the same mistakes happen for all compounds. The big problem arises when the absolute measurement from the NMR method is compared with the relative results of the HPLC method.

In that case, on top of all the above problems there is a very simplistic but widespread confusion: Tyrosol has a molecular weight of 138 mg/mmol, while oleocanthal has a molecular weight of 304 mg/mmol, oleacein 320 mg/mmol etc. So, when we measure an olive oil by NMR containing 304 mg/Kg of oleocanthal, it means that it contains 1 mmol/Kg of oleocanthal. If we assume that one molecule of oleocanthal absorbs the same amount of light as one molecule of tyrosol (which is not exactly true) then if we express the results as tyrosol equivalents, then this amount of 1 mmol corresponds to 138 mg/Kg of tyrosol. It is astonishing when chemists are trying to compare absolute measurements (like NMR) with relative measurements expressed in equivalents (like HPLC), without explaining this basic difference. Simply the difference of the molecular weight by itself can explain why the HPLC values are generally much lower than the NMR. If besides this problem we add the underestimation by HPLC of the compounds bearing the tyrosol moiety, then it is obvious that HPLC measurement is completely inappropriate and misleading for the measurement of independent phenolics and in addition it is obvious why it gives lower arithmetic values in comparison with NMR.

For all the above reasons the comparison among different olive oil concerning their total phenolic content should be performed only by the same method. Especially the measurement of independent phenolic compounds can be reliably measured only by NMR (or appropriately calibrated LCMS) but not by HPLC-UV with tyrosol equivalents.

It should also be mentioned that the EU heath claim is certified using absolute measurements of phenolics expressed as mg/Kg and not as tyrosol equivalents (even if they are also expressed as mg/Kg).