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EDITORIAL

Globalization of the trade of agricultural products contributed significantly to the discussion about potential hazards involved, thereby increasing especially the awareness for mycotoxins.
Approximately 300 to 400 substances are known as mycotoxins produced by various mould species on many agricultural commodities and processed food and feed.
The analysis of mycotoxins became an issue of global interest, in particular because most countries set up regulative limits or guidance levels for the tolerance of such contaminants in feed and food commodities and products thereof.
Besides rapid analysis methods, like ELISA (Enzyme Linked Immunosorbent Assay) and LFD (Lateral Flow Device), multitoxin methods using HPLC-MS (High Performance Liquid Chromatography-Mass Spectrometry) become more and more important. Mass spectrometry enables the determination of more than 200 mycotoxins within one run. This powerful tool is often limited by matrix effects during ionization in the MS source. There are several possibilities to overcome these effects, e.g. the addition of internal standards (IS) to the sample. Internal standards are stable isotope labelled molecules of the target analyte. Due to this fact the IS has the same physicochemical properties and an identical molecular structure as the naturally occurring analyte.

Markus Kainz

Multi-Mycotoxin testing
A routine approach

Modern Mycotoxin Analysis

High performance liquid chromatography (HPLC) and gas chromatography (GC) have traditionally been the method of choice when it comes to analysis of mycotoxins and sensitive, reliable results are required with minimum variability.

HPLC systems can be coupled with various detectors, e.g. spectrophotometric detectors (UV-Vis, diode array), refractometers (RI), fluorescence detectors (FLD), electrochemical detectors, radioactivity detectors and mass spectrometers depending on the field of activity.

For the analysis of mycotoxins the coupling of liquid chromatography (LC) and mass spectrometry (MS) provides a great potential.

Within this combination some disadvantages are shown but they are mainly overcome by the advantages (*see Table 1*).

Table 1 *- Advantages and disadvantages of LC-MS/MS systems*

The Pros	The Cons
Simultaneous detection of different analytes	Expensive instrumentation and trained staff needed
Over 200 different mycotoxins and fungal metabolites within 1 run	Ion suppression/enhancement leads to different signal intensities between calibrants and matrix sample
Simplified sample preparation	Matrix influence on ionisation process within the mass spectrometer
No derivatization	Potential source of systematic errors, limited accuracy and repeatability in quantitative analyses
Selective and sensitive detection method with tandem MS systems	

There are several possibilities to improve the accuracy and sensitivity of the system. One way would be a sample clean-up prior to analysis and the addition of internal standards to the sample.

Clean-up & MycoSpin™

For the analysis with LC-MS/MS different, frequently used sample preparation methods exist, e.g. dilute and shoot method without clean-up, the SPE (solid phase extraction) clean-up and the IAC (immuno affinity column) clean-up. As an additional method Romer Labs® offers Multi-Mycotoxin clean-up columns named MycoSpin™.

The MycoSpin™ is a dispersive SPE in spin column format containing optimised packing material for mycotoxins and allows the simultaneous clean-up for several mycotoxins. Compared to the more cost intensive IAC, the MycoSpin™ gives a good alternative. The columns are storable at room temperature and are not limited to one mycotoxin. The general workflow of the MycoSpin™ is shown in *Figure 1*.

Diverse trials show a good recovery for several toxins and commodities (displayed in *Table 2* and *Table 3*). The recoveries for corn and peanut are shown exemplarily in *Figure 2*.

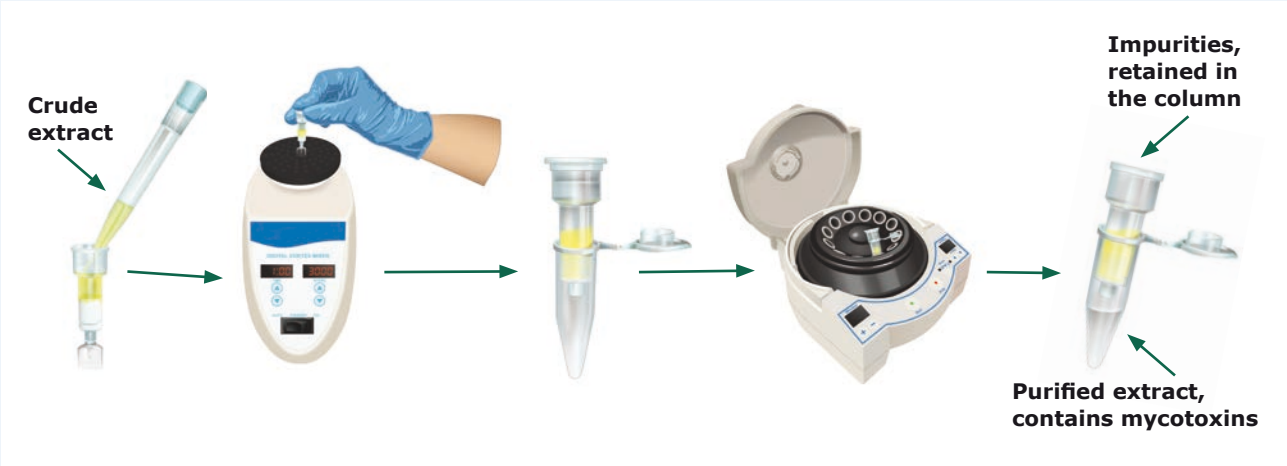

Figure 1. *General Workflow*

Table 2 *- Commodities tested with MycoSpin™*

Commodity
Barley & Wheat
Corn & Corn Gluten Meal
Distillers Dried Grain
Peanuts, Rice, Soy
Finished Feed
Mustard

Table 3 *- Toxins tested with MycoSpin™*

Toxin
Zearalenone
Type A-Trichothecenes
(T2, HT2, NEO, DAS)
Type B-Trichothecenes
(DON, Acetyl-DON, FusX, NIV)
Aflatoxins
Ochratoxin A
Fumonisins

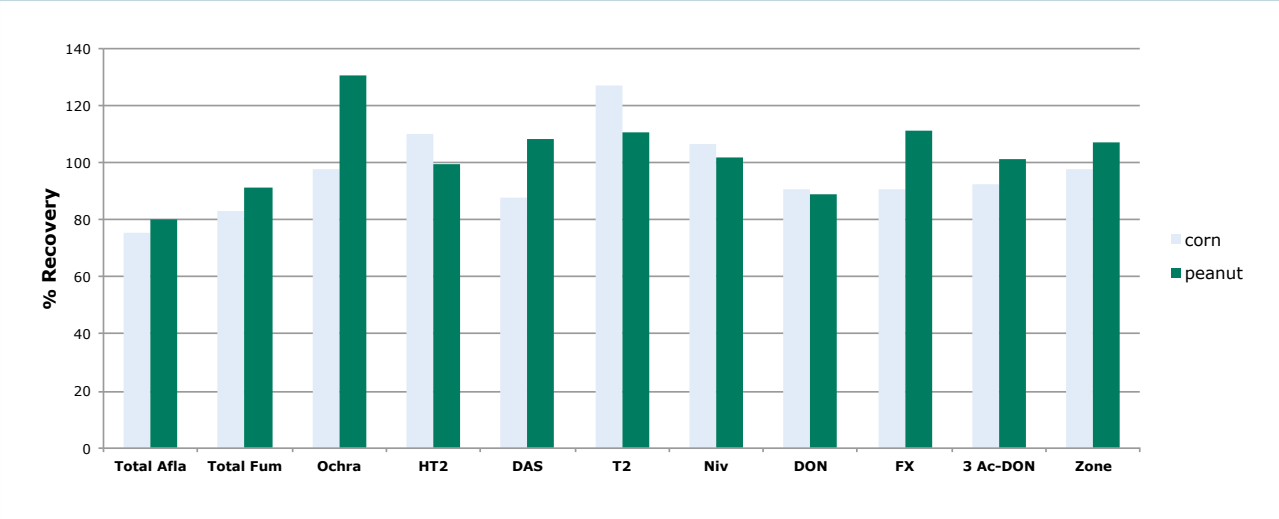

Figure 2. *Recoveries for different toxins in corn and peanut*

Matrix effects

Matrix effects in the LC-MS/MS are difficult to control. Matrix effects result from co-eluting residual matrix components which affect the ionisation efficiency of target analytes and can lead to erroneous results. They can cause an ion suppression leading to an under-estimation of the target analyte or an ion enhancement, which causes an over-estimation of the target analyte, examples are displayed in *Figure 3*. The impact of matrix effects differs from analyte to analyte and from one commodity to another.

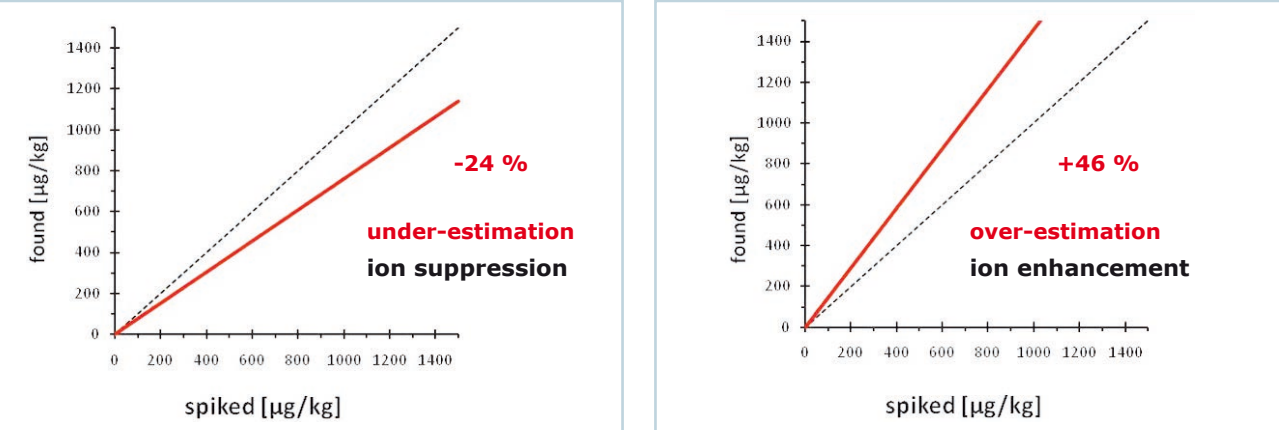

Figure 3. *Example for matrix effects for DON and T-2 Toxin in corn*

Internal Standards – Usage & Costs

^{13}C-isotope labelled mycotoxins are one application of an internal standard (IS) used in mass spectrometry. All carbon atoms in the molecule are substituted by the stable carbon isotope ^{13}C (*see Figure 4*).

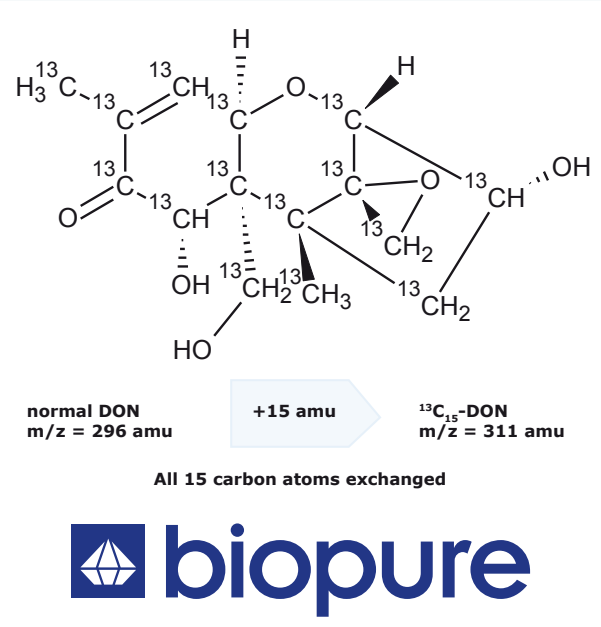

Figure 4. *Chemical structure of $^{13}C_{15}$ Deoxynivalenol*

Because of similar chemical behavior of analyte and ^{13}C analog, these substances behave similar in chromatography but differentiate in mass spectrometry. Recovery losses from sample preparation and ion suppression or enhancement effects in the MS source can be eliminated.

Application of Internal Standard (IS):

There are different approaches how to use an internal standard. The most effective method is to apply the IS onto the homogenized sample prior to extraction. Another approach is the addition of IS after the extraction or prior to HPLC analysis.

The different application methods (*see Figure 5*) have benefits, but to choose the "best approach" several points need to be considered. For example an important factor is the variety of samples analysed on a regular basis. In general, third party laboratories analyze a high number of versatile samples on a daily basis. A validation of different commodities is very time consuming and cost intensive. Each commodity needs to be validated in detail and recovery has to be determined as well. Thus the routine method has limited flexibility regarding "unknown" commodities which are not validated.
The usage of IS prior to extraction will overcome the matrix effect and compensate also possible losses during extraction or clean-up.

For commodities which are analysed almost every day matrix validations might be useful. Therefore a point of addition of IS closer to the LC-MS/MS analysis can be considered to compensate the matrix effect only.

A more cost effective approach is the addition of IS after the extraction or prior to HPLC analysis. Both solutions require a thorough validation of each commodity and calculation for recovery.

Cost Calculation

The price per sample is crucial for the decision how to use the internal standard (IS), but a general calculation is difficult due to several aspects: sensitivity of the instrument, sample weight, volume of extraction solvent, clean-up procedure, sample concentration, injection volume. All factors mentioned will influence the cost calculation.

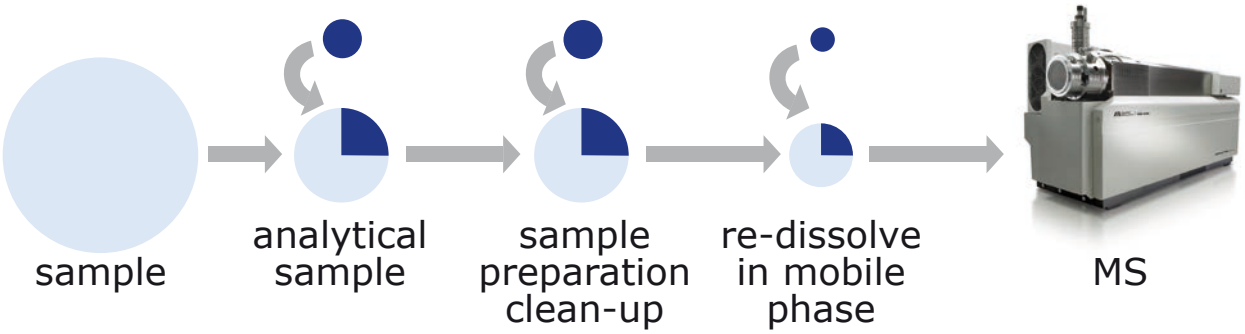

Figure 5. *different approaches of ^{13}C IS application*

Enclosed *Table 4* and *Table 5* show an example of ^{13}C IS concentrations which can be used. The method requires the preparation of a positive mode and a negative mode internal standard solution. The calibrated values are based on the sample preparation and the sensitivity of the LC-MS/MS system used.
In this case the point of addition of the calibrant mixture to the sample will be after clean-up procedure using MycoSpin™.

Table 4 *- Positive Mode – Amounts of Internal Standard Solution*

Internal Standard	Standard Concentration [µg/mL]	Calibrated Value [ppb]
Aflatoxin B1	0.5 each	1.25 each
Aflatoxin B2		
Aflatoxin G1		
Aflatoxin G2		
Fumonisin B1	25	250
Fumonisin B2	10	80
Fumonisin B3	10	80
HT-2 Toxin	25	250
T-2 Toxin	25	250
Diacetoxyscirpenol	25	75
Ochratoxin A	10	2.5

Table 5 *- Negative Mode – Amounts of Internal Standard Solution*

Internal Standard	Standard Concentration [µg/mL]	Calibrated Value [ppb]
Deoxynivalenol	25	250
Nivalenol	25	250
3-Acetyl Deoxynivalenol	25	250
Zearalenone	25	25

The mixture of ^{13}C IS, each positive and negative mode solution, is prepared in 25 mL of solvent (mobile phase). Taking into account a requirement of 75 µL for each sample, the solution will last for more than 300 analyses. Using this approach together with a MycoSpin™, the price/sample will be € 12.5 Euro. Other methods may result in different cost.
Figure 6 and *Figure 7* show the chromatograms of the positive mode and the negative mode.

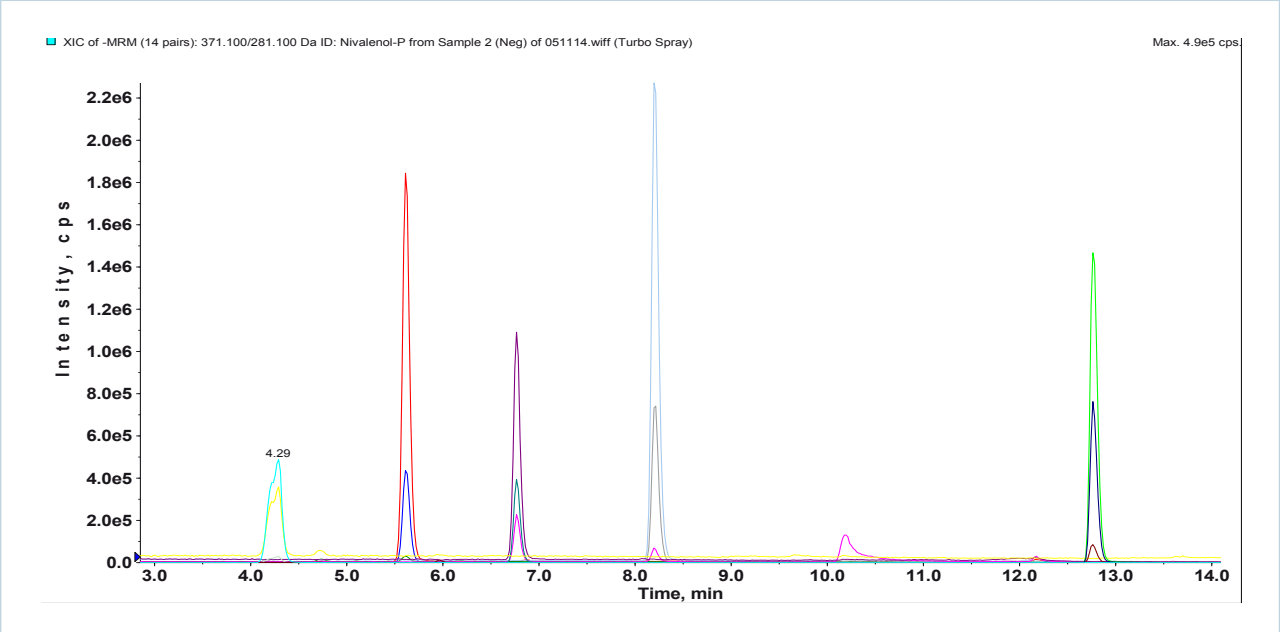

Figure 6. *Chromatogram of negative mode*

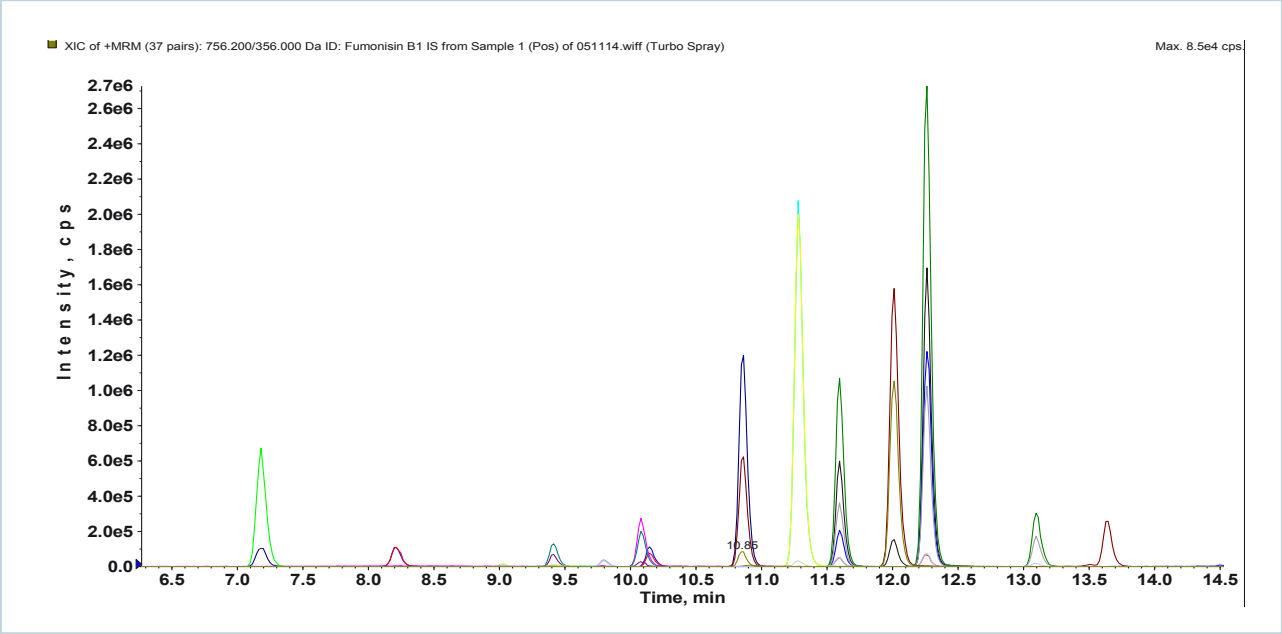

Figure 7. *Chromatogram of positive mode*

Multitoxin method – Romer Labs® Routine Method (an example)

Instrument:	Applied Biosystems
5500 QTrap LC-MS/MS System	
Mobile Phase A:	Water with 2 mM Ammonium Acetate and 0.5 % Acetic Acid
Mobile Phase B:	Methanol with 2 mM Ammonium Acetate and 0.5 % Acetic Acid
Method Runtime:	16 min
Injection Volume:	20 µL
Flow Rate:	1 mL/min
Column Temperature:	40 °C
Amount of ISTD added:	from 0.2 to 40 ng depending on the mycotoxin
Number of mycotoxins detected:	15
Number of ISTD added:	15
Point of ISTD added:	after clean up
Gradient:	
Min	% B
0 – 2	10
2 – 14	10
14 – 15	97
15 – 15.1	10
15.1 – 16	10

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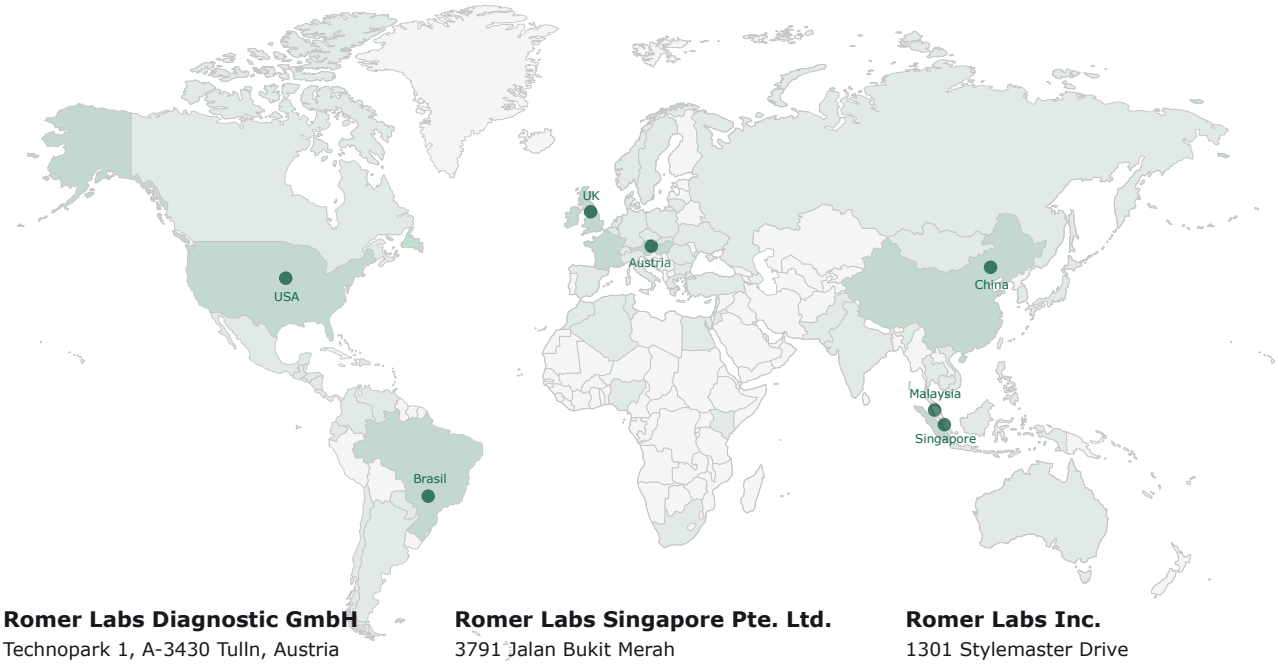

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