B&W TEK
A Metrohm Group Company

Technical Note
Method Development with NanoRam®-1064

General Scope

The NanoRam®-1064 is equipped with a patented reduced variable multivariate (RVM) algorithm that allows for robust, rapid and nondestructive raw material identification (RMID). The method compares the tested material Raman spectrum with the reduced variates in the method model, and provides a statistical p-value with a pre-defined threshold for pass/fail result on whether the tested material is accepted as a member of a class. Although the process of building, validating and using a method is well-defined through software, the robustness of the method is dependent on proper practice of sampling, validation, and method maintenance. In this document, we will detail the recommended practices for using the multivariate method with NanoRam-1064. These practices are recommended for end users who are in the pharmaceutical environment, and can expand to other industries as well. This document is not a standard operating procedure, but is aimed to serve as a general reference for NanoRam-1064 users who would like to build an SOP for method development, validation and implementation. Users are recommended to create their own standard operating procedure, following our recommendations.

Method Building

Chemical Suitability

Determining the chemical suitability of the raw material for identification by Raman is the first step towards building a method. Generally, Raman is suited to identify and analyze organic and inorganic molecules that exhibit a significant change in polarizability upon vibration. These materials typically include active pharmaceutical ingredients, excipients, coatings, polyatomic inorganic molecules, solvents, etc. However, some of these molecules generate fluorescence when excited with a 785-nm or 830-nm laser, which can reduce or even completely overwhelm the Raman signal that can be used for identification. The NanoRam-1064 is equipped with a 1064-nm laser, which can more effectively mitigate fluorescence generated from the visible range of the electromagnetic spectrum, allowing the possibility to identify colored material, fluorescent coatings, natural derivatives, and biomaterials.

Aside from pure raw materials, some users may want to also identify different mixtures, polycrystalline samples and polymorphic samples. Because of the inhomogeneity of these materials that are not of one pure form or composition, creating a robust method can be a challenging task due to the large variance in their molecular structure. This leads to a large variance of the sample spectra measured in different locations on the sample. We will address the solution for this problem in the later sections.

Certainly, there are materials that are not suitable for Raman analysis. Black-colored material, metals, and mono-atomic salts (e.g. NaCl, etc) are inherently Raman inactive. Water, thin-walled plastics and glass, also exhibit a weak Raman signal. Some natural products and dark-colored materials may also generate fluorescence which cannot be mitigated even with a 1064-nm laser. In these cases, alternative methods such as FTIR or LC/GC-MS are recommended for sample identification.

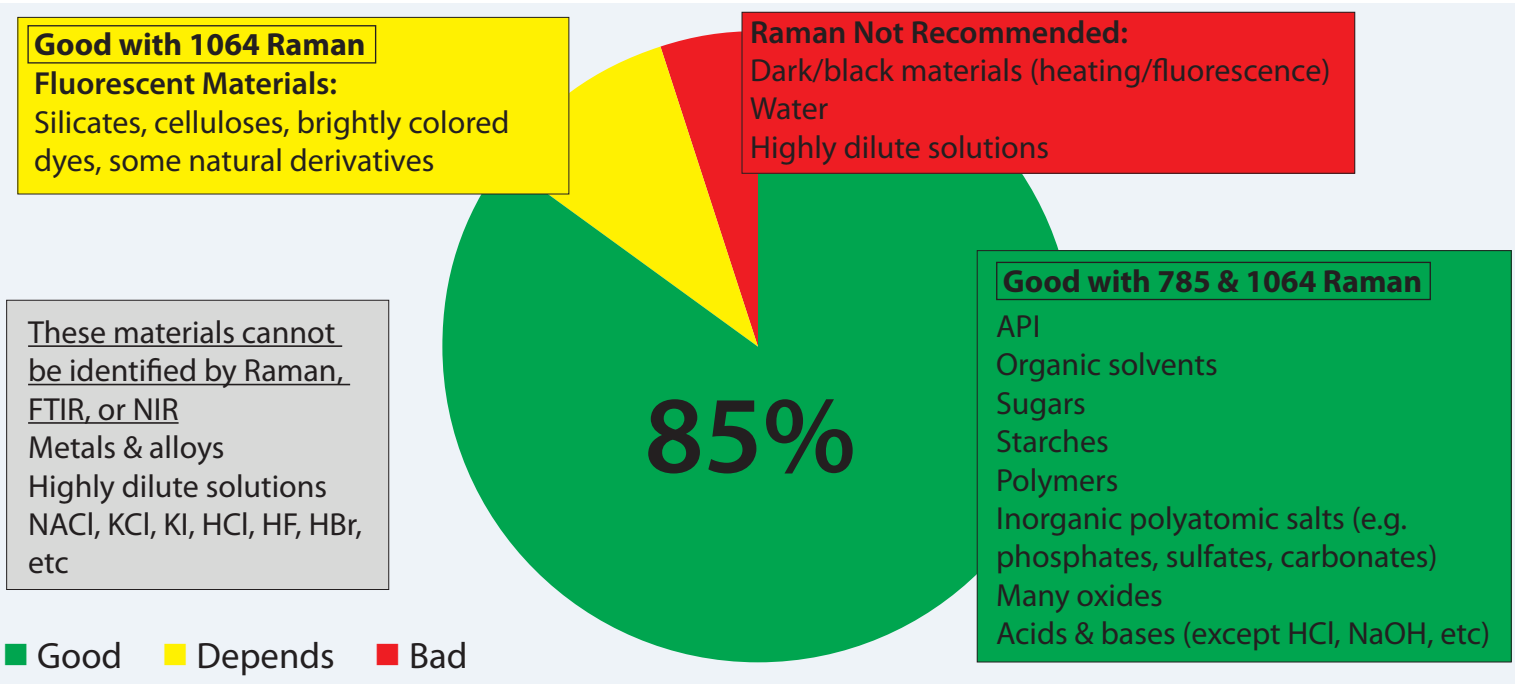

Figure 1. Chemical Suitability for Raman

Library vs. Method

Determining the chemical suitability of the raw material for identification by Raman is the first step towards building a method. Generally, Raman is suited to identify and analyze organic and inorganic molecules that exhibit a significant change in polarizability upon vibration. These materials typically include active pharmaceutical ingredients, excipients, coatings, polyatomic inorganic molecules, solvents, etc. However, some of these molecules generate fluorescence when excited with a 785-nm or 830-nm laser, which can reduce or even completely overwhelm the Raman signal that can be used for identification. The NanoRam-1064 is equipped with a 1064-nm laser, which can more effectively mitigate fluorescence generated from the visible range of the electromagnetic spectrum, allowing the possibility to identify colored material, fluorescent coatings, natural derivatives, and biomaterials.

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Adaptors

As shown in Figure 2, the NanoRam-1064 is equipped with numerous adaptors for optimal measurement of different sample types. The general guideline for which adaptor to use for RMID can be summarized in two steps:

1. Choose the best-suited adaptor for each raw material and packaging; and
2. Keep consistent adaptor usage across method building, method validation and method implementation.

Each adaptor may be used in different sampling scenarios:

a. **Point and shoot adaptor:** Most commonly used adaptor for identifying samples. With a working distance slightly less than 3mm, it can scan through thin plastic packaging for powders, gels and solids.

b. **Right-angle adaptor:** Also used for identifying solids, with the advantage that it can be used for hands-free measurements.

c. **Bottle adaptor:** Has a working distance around 5mm, and is dedicated for detecting liquid and solid within clear or translucent plastic bottles or amber glass bottles.

d. **Liquid vial holder:** Dedicated to detect liquids within 10mm-15mm diameter borosilicate glass vials.

e. **Immersion probe:** The immersion probe has a working distance near contact (<1mm) and it is suitable for immersing into different solid/liquid for quick and easy raw material detection inside a drum/container.

f. **Polystyrene cap:** Used to validate the instrument performance and Raman shift calibration.

Figure 2. NanoRam-1064 and Adaptors (standard and optional)

The NanoRam-1064 also has some optional adaptors that can be purchased as add-ons.

g. **The large bottle adaptor:** With a working distance around 10mm, it is dedicated for detecting liquid and solid within thick transparent plastic bottles and amber glass bottles.

h. **The large spot adaptor**: Uses defocused light to collect the Raman spectra. The signal from the sample is then increased with the built-in optical reflective cavity at the tip of the adaptor. Because of the significantly reduced laser intensity density, and a much larger sampling area compared to the focused adaptor, this adaptor is particularly suited for powdered samples that are light-sensitive, or mixture samples such as cell culture media, samples of mixed polymorphic form and polycrystalline samples.

Once it is established that a good spectrum can be acquired reliably using an adaptor, it is important to keep using the same adaptor for the rest of the method development process, including method validation and method implementation. This is important because a method built with one type of adaptor may have different sensitivity and specificity than when used with a different adaptor,

which can lead to reduced robustness and false negative results. The method variability should be built on the raw material differences with minimal variation in sampling.

Figure 3 is an example which demonstrates the significance of the large spot adaptor for more representative sampling of heterogeneous samples. Figure 3a shows the spectra from a method of a powdered cell-culture media sample built with the point and shoot adaptor. The 28 collected spectra show a large amount of variance as measurements are made on different areas of the sample. This method failed validation which is to be expected, as the sample itself is a mixture and contains a large amount of variance from one location to another when collected over a small area with the point and shoot adaptor, which only has a spot size of less than 0.2 mm in diameter. In this situation, a more reliable method can be built with the large spot adaptor, which interrogates a much wider area on the sample (~4 mm in diameter) compared to the point and shoot adaptor. Figure 3b shows the 28 method spectra of the same sample collected with a large spot adaptor where localized variation is not as pronounced. The method spectra are more consistent, resulting in a lower variance and a passing method validation. However, it is important to note that the validation of this method will fail if the validation spectra are collected using a different adaptor, such as the point and shoot adaptor or the right-angle adaptor.

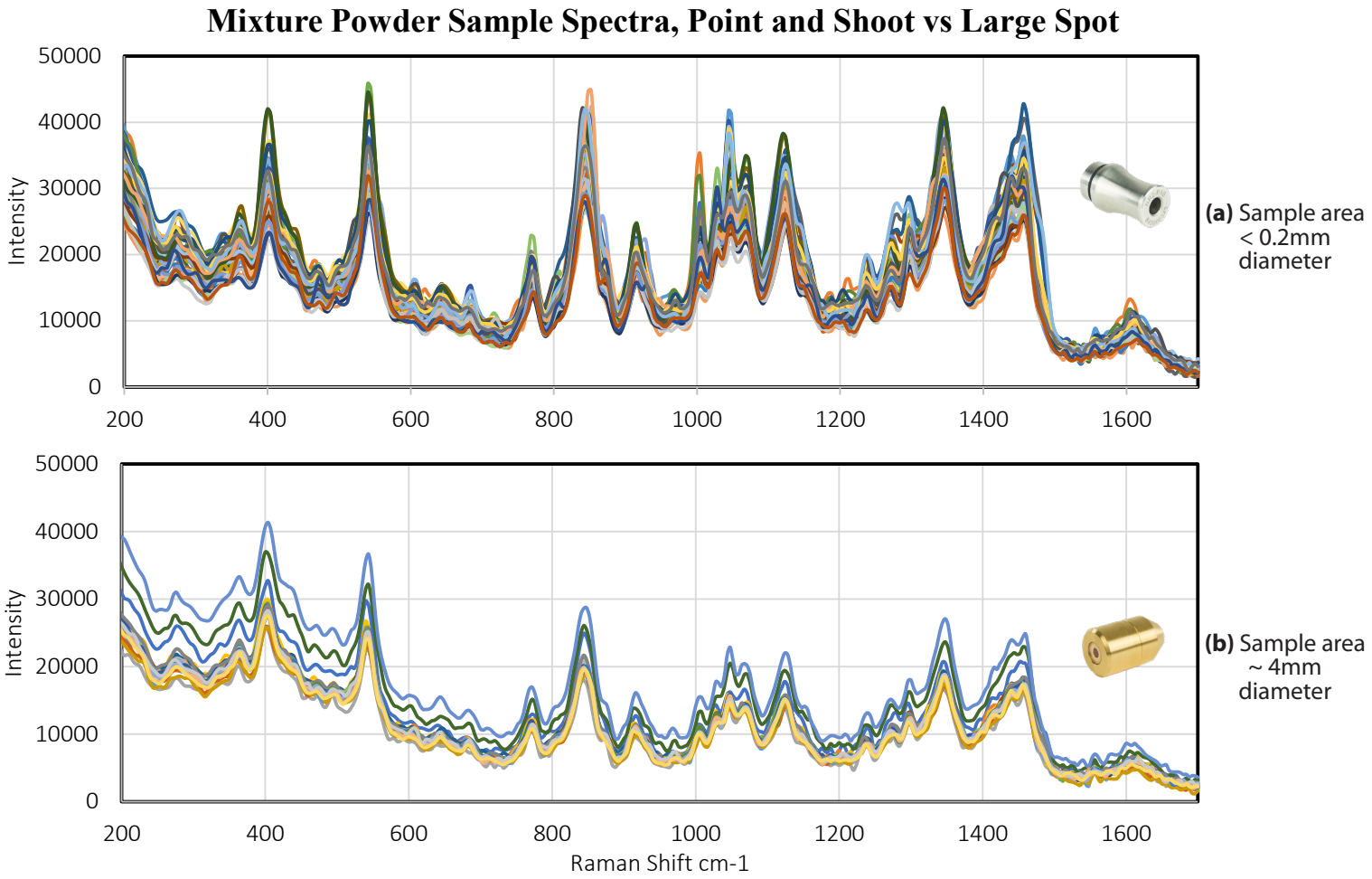


Figure 3. Sets of method spectra collected from a powdered cell-culture mixture sample using (a) a point and shoot adaptor and (b) a large spot adaptor.

Including Representative Spectra

The most important factor for building a reliable method using NanoRam-1064 is that the method include a sufficient amount of representative spectra. Specifically, these conditions should be considered:

a. The developer is encouraged to build the method with samples that are verified with the primary identification method. These primary identification methods include certifications from the supplier, NIR/FTIR, advanced chromatography characterizations, etc.

b. The developer should include samples from different lots and different vendors while building the method. This is recommended as minor deviations from different lots and vendors may give spectral differences that affect the robustness of the method.

c. The developer is also encouraged to include spectra collected by different end users. This is recommended as different users might have different techniques of collecting the spectra. We encourage users to reference our user manual for the recommended sampling practices.

d. Other variances that are not related to the chemical itself, but are expected in routine operations should also be considered.

The exact number of spectra needed to build a robust method depends on the amount of variance the sample has. The larger the variance, the more representative spectra are needed. The NanoRam-1064 software includes 5 spectra as default when building a method and the user can choose to add more spectra into the method prior to method validation.

As shown in Figure 4, the NanoRam-1064 software will display the HQI value of the collected method spectrum against the average of the previously collected set of method spectra. Ideally, the user should include an equal proportion of low HQI value spectra and high HQI value spectra. However, for spectra which have a reported HQI value lower than 80 we encourage users to re-examine the sample or sampling process before proceeding to ensure this is an acceptable sample and sample spectrum.

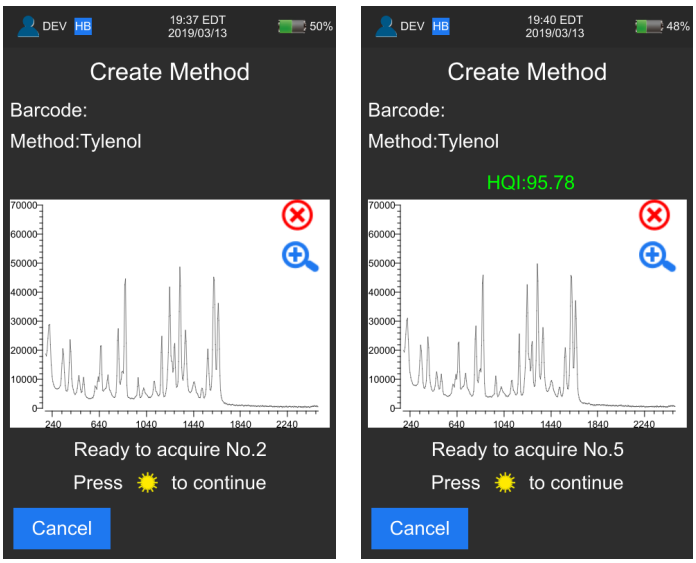


Figure 4. NanoRam-1064 Method creating interface. HQI value of each collected method spectrum is displayed starting from the second collected spectrum.

Packaging Material

Packaging materials can affect the performance of the method. Thin-walled plastics and glass containers are recommended as packaging materials because they are weak Raman scatterers, meaning spectra of the samples can be measured inside of these materials with minimal interference. The packaging material chosen for a given method should be considered prior to method creation. If the packaging that the raw material arrives in is not suitable to measure through, then re-sampling into another container such as a plastic bag or glass vial may be necessary.

The samples measured against the method should be in the same packaging material as was used to create the method. The thickness and number of layers of a plastic bag may affect the robustness of the method. Figure 5 shows spectra of acetaminophen acquired using different layers of clear polyethylene bags (0.1 mm in thickness) compared with the spectrum of a pure polyethylene bag. As the number of layers of polyethylene increases, the spectral contribution from the polyethylene increases due to the packaging, and not the material that is being tested.

In this case, acetaminophen measured through one and two layers of polyethylene bags did not fail the acetaminophen method, but acetaminophen measured through three polyethylene bags did fail the acetaminophen method due to an increase in the contribution from the polyethylene packaging material. The sample material and the packaging material should remain consistent and should be tested prior to creating the method to note any interference from the packaging material.

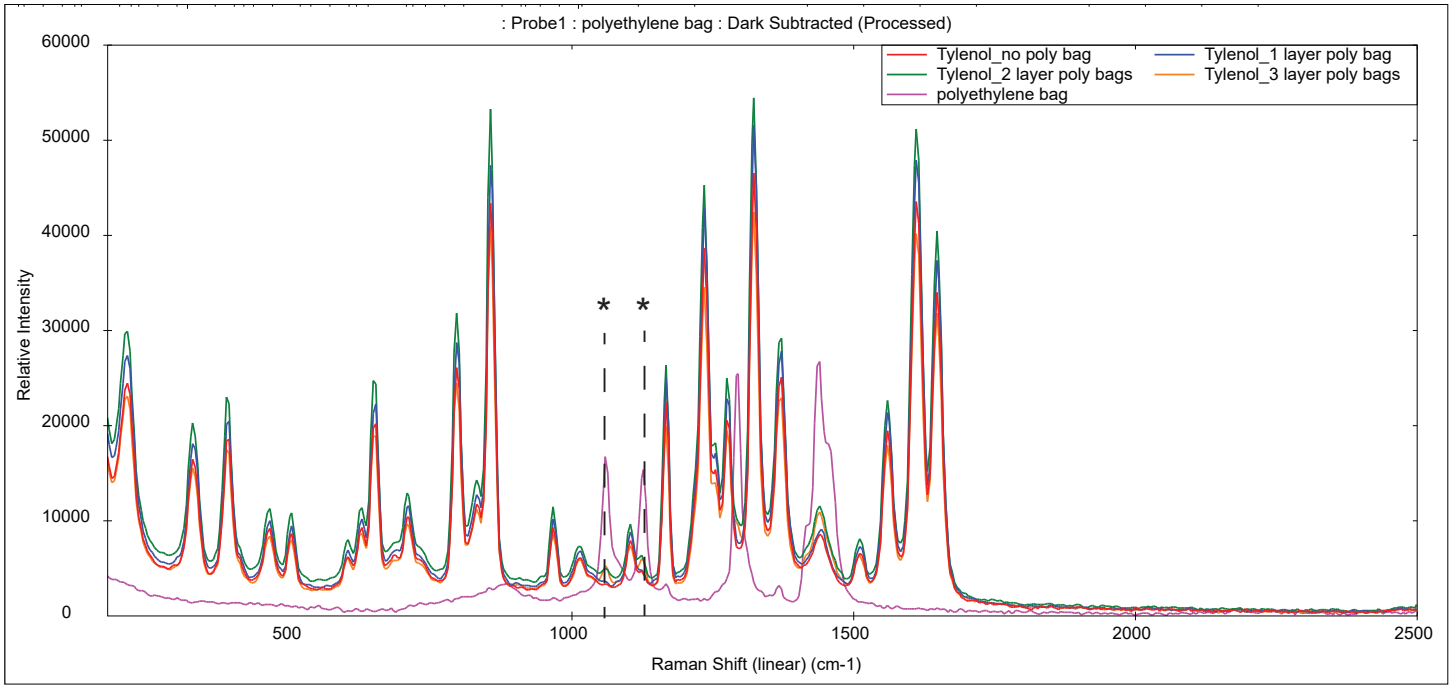


Figure 5. Raman spectra of acetaminophen measured through different layers of polyethylene bags (each ~0.1 mm thick) compared with pure polyethylene. As the number of layers increases, the spectral contribution from the polyethylene is stronger, particularly the peaks at ~1060 cm^{-1} and 1130 cm^{-1}, denoted by an asterisk.

Method Validation

General Requirement

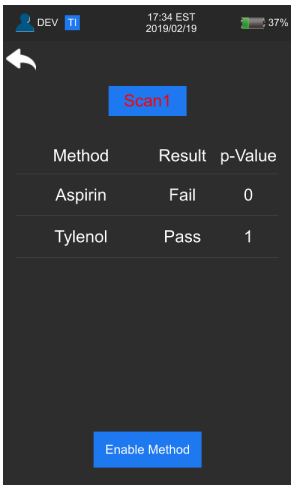
NanoRam-1064 is designed to perform identification tests (category IV) as detailed in USP <1225> and ICH Q2. The specificity requirement for method validation as defined in ICH Q2 is:

> *"the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc."*

Therefore, both positive sample and negative samples should be used to ensure the validity of the method.

The NanoRam-1064 software has an on-board method validation function. A new method must be validated before it can be enabled in the software. The onboard software requires the user to acquire between one and three positive samples for this method, and then cross validate these spectra against all of the enabled methods on the system (Figure 6a). After synchronizing the unit with NID EX, a method cross validation table can be examined at the "Cross Validation" ➔ "Report" section, as shown in Figure 6b. A fully validated method must pass all validation scans of itself and fail all validation scans of all the other methods.

a. NOS On-board Method Validation



b. NID EX Method Cross Validation

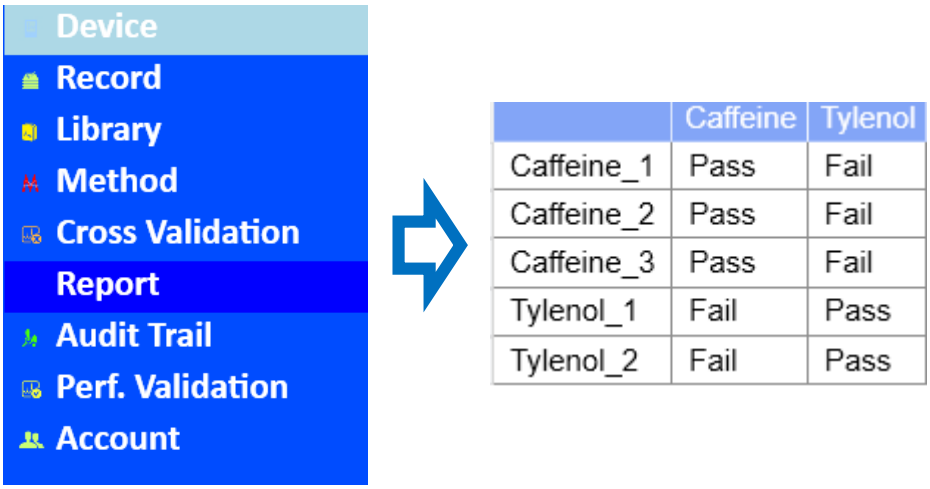


Figure 6. NanoRam-1064 software functions for method validation

Challenges

Comparing to state-of-the-art benchtop devices, handheld devices typically have reduced throughput, resolution, spectral range and signal-to-noise level. Therefore, a lack of specificity might be observed with samples that have with similar molecular structures and Raman spectra, especially among non-pure samples. There are a few steps a method developer can do to improve the specificity of the method:

a. Examine the spectra included in the method. Ensure the spectra correctly represent the sample material, and do not contain any unwanted contaminating signal from the packaging material.

b. Compare the method spectra with the negative sample spectra. Ensure the variation between the method and the negative sample is bigger than the spectral variation of the method itself. In other words: If the method spectra are too noisy, this noise may be stronger than the minor differences of the spectra of the samples, so the differences may not be detectable.

c. Adjust the spectral range to highlight the area where significant differences in spectral signature are present. To do so, the user must be aware of the risk that the highlighted area might not encompass the entire spectral signature. Therefore, this technique must be implemented with caution and method validation especially of structurally similar materials should be conducted.

d. Adjust the p-value threshold. The user should be aware that changing the p-value threshold will change the statistical confidence of the "Pass" and "Fail" result. Our recommended p-value threshold is 0.05, which corresponds to a 95% statistical confidence interval. Increasing the p-value threshold corresponds to a reduction in this confidence interval, which may increase the chances of a false negative result; decreasing the p-value threshold corresponds to an increase in the confidence interval, which may increase the changes of a false positive result.

Method Implementation and Interpretation

General Steps

The NanoRam-1064 software has an Identification mode that uses validated methods for raw material identifications. As shown in Figure 7, the built-in workflow allows users to choose the number of samples for each run, choose the method to test the sample either manually or by scanning a barcode, and enter crucial tracking information such as Run Name, Product Name, Barcode, Supplier, etc. After the scan finishes, the method will show a "Pass" or a "Fail" result with a statistical confidence p-value for reference.

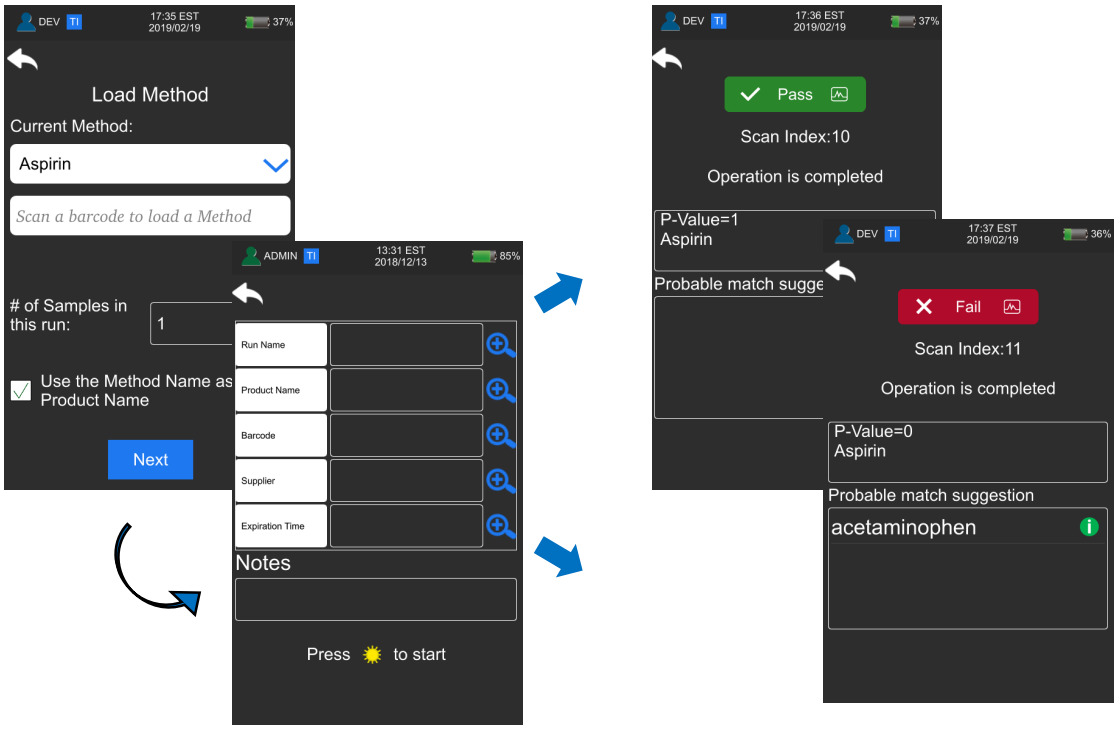


Figure 7. Identification using the "Aspirin" method and the Pass and Fail results

Data Management

Data collected on the NanoRam-1064 are synchronized to the NID EX database, where the method spectra and cross validation matrix and reports can be viewed. Figure 8 shows a method report for Tylenol generated by NID EX. The method validation report shows the measurement parameters and the validation scan results. The cross-validation table can also be viewed and exported to a CSV file. Figure 9 shows the exported method cross-validation table for validation scans of cellulose, ethanol, and gelatin methods. The spectra files can also be exported for further review. Both scan data and method data can be exported as SPC and CSV files. The SPC files are compatible to view

in BWSpec® software, and CSV files can be opened and graphed in Microsoft Excel. In the case that data needs to be sent to B&W Tek Support for review, please provide data in both SPC and CSV file formats.

Figure 8. Method report exported from NID EX

	A	B	C	D
1		Cellulose	Ethanol	Porcine gelatin
2	Cellulose_1	Pass	Fail	Fail
3	Ethanol_1	Fail	Pass	Fail
4	Porcine gelatin_1	Fail	Fail	Pass
5				
6		Cellulose	Ethanol	Porcine gelatin
7	Cellulose_1	1	0	0
8	Ethanol_1	0	1	0
9	Porcine gelatin_1	0	0	1

Figure 9. Cross-validation table exported from NID EX as a CSV file

Practical Considerations

In the scenario where the identification of raw material returned a failed result, it is good practice to carry out root cause analysis for these out-of-specification (OOS) results. Items to consider in determining the root cause can follow the recommendation from USP <1858> for method validation, which lists a series of conditions where method revalidation may be needed. These following conditions may cause a sample to fail a method:

a. The sample measured is the wrong material.

b. The sample identified is from a different supplier/vendor/batch and exhibits variation that was not included in the original method.

c. The sample's packaging material has changed.

d. Different operators' sampling has contributed to a change of relative peak intensity (i.e. one operator presses harder on the sample bag than another, causing a change in the ratio of Raman peaks of the sample of interest compared to the packaging).

e. A different accessory was used to measure the sample compared to the accessory used to create the method (e.g. using a liquid vial adaptor to measure a sample when an immersion probe was used to create the method).

f. The accessory was not fully pushed in.

g. The lens shaft is smudged or dirty. Fingerprints are the biggest culprit- clean with isopropyl and a Kimwipe or clean cloth.

h. Did the instrument undergo repair?

i. Was the method transferred from another instrument but not properly validated?

Summary

In this technical note, we summarized the general guidelines and recommendations for method building using NanoRam-1064. The primary purpose of this article is to give the user a full understanding of the entire scope of method building, the compliance requirement behind it and to help answer the common questions a user might have. We hope that the information presented here will help the end users understand the method building process, defining their own method building procedure and build reliable and robust methods.

References

1. B&W Tek, LLC (2019). *Reduced Variable Multivariate Analysis for Material Identification with the NanoRam®-1064* (Application Note 410000044), https://bwtek.com/appnotes/reduced-variable-multivariate-analysis-for-material-identification-with-the-nanoram-1064/.

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