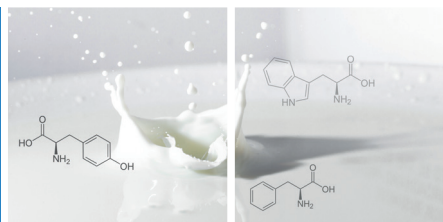


Raman  
Spectroscopy,  
Granulometry &  
Fluorescence

## Milk compounds characterization by optical spectroscopies and laser diffraction



Application  
Note  
Multitechniques  
AN 04

Thibault Brulé, Nicolas Buton & Alessia Quatela, Application Engineers, HORIBA Scientific, Palaiseau, France

**In the food industry, the compounds characterization is a critical step to ensure the quality of the products or to provide information to customers which can be sensitive to allergies. In this application note, we showed how optical spectroscopies and laser diffraction can help for food compounds characterization, especially on a specific product, i.e. milks.**

**Keywords:** Raman spectroscopy, Granulometry, Fluorescence spectrophotometry, A-TEEM, Food & Beverages, Milk composition.

### Introduction

In the food industry, a critical step concerns the quality control of the products to determine the level of the different chemical compounds. Indeed, as more and more people over the world are subject to food allergies and/or restrictive regimes, it is essential to control the food quality and to characterize the compounds. One of the main consumer and critical products in the market is milk. Milk is an important source of allergies and its composition must be monitored especially in case of restrictive regimes due to its richness. Milk can be characterized by using a combination of Particle Size Analysis, Raman spectroscopy and Fluorescence spectroscopy. Each of these techniques provides information about milk chemical composition such as fatty acid, vitamins, etc.

In this work, we apply these three analytical techniques on different kind of milks. First, we characterized cow milks at different fat levels. Then, we analyzed and compared them with milk alternatives from different origins, i.e. soy and baby powdered milks.

### Instruments and methods:

Particle Size analysis is based on laser diffraction. The central idea in laser diffraction is that a particle will scatter light at an angle determined by its particle's size. Larger particles scatter light at small angles while smaller particles scatter light at wide angles. A collection of particles produces a pattern of scattered light defined by intensity and angle that can be transformed into a particle size distribution result. The system used for this work is a HORIBA LA-960. It combines

the most popular modern sizing technique with state-of-the-art refinements to measure wet and dry samples measuring from 10 nanometers to 5 millimeters.

Raman Spectroscopy is a non-destructive chemical analysis technique which provides detailed informations about chemical structure, phase and polymorphism, crystallinity and molecular interactions. It is based upon the interaction of light with the chemical bonds within a material. Raman is a light scattering technique, whereby a molecule scatters incident light from a high intensity laser light source. Most of the scattered light is at the same wavelength (or color) as the laser source and does not provide useful information – this is called Rayleigh Scatter. However, a small amount of light (typically 0.0000001%) is scattered at different wavelengths (or colors), which depend on the chemical structure of the analyte – this is called Raman Scatter. The system used is a HORIBA XploRA™PLUS. It incorporates unique and powerful functions in a reliable, high performance system, ideally suited to the research and analytical lab. It is fully confocal, not compromising image quality, spatial or depth resolution.



Figure 1. Experimental systems used for these experiments. Left: LA-960 Particle Size Analyzer. Middle: XploRA™PLUS Raman micro-spectrometer. Right: Aqualog spectrofluorometer.

This study employs the patented method of simultaneous Absorbance-Transmission and fluorescence Excitation-Emission Matrix (A-TEEM™) spectroscopy with the Aqualog® from HORIBA Instruments Inc. The Aqualog® optical bench includes a special aberration-corrected double-grating excitation monochromator, a reference detector and absorbance detector (both Si photodiodes), and a unique emission detector comprised of a thermoelectrically cooled back-illuminated CCD and spectrograph. The system incrementally scans excitation from high energy to low energy and can collect the full emission spectrum at each excitation increment to rapidly and simultaneously generate absorbance spectra and excitation-emission spectral maps or matrices. The Aqualog®-coupled software is equipped with a built-in tool for normalization to water Raman scattering or quinine sulfate units for the defined emission conditions, correction for the influence of Inner Filter Effect (IFE) and Rayleigh masking.

## Cow milks characterization

In order to monitor the composition of milk at different fat levels, five different cow milks were analyzed. The first one was a raw milk, directly originating from cows and collected in a farm. The four others were commercial milks with different fat levels (whole, semi-skimmed and skimmed). The two semi skimmed milks were from two different brands.

Using LA-960®, particle size analyzes of these four milks were performed to obtain the particle size distributions of the liquid samples. The size distributions are shown in Figure 2. In theory, the particles present in milks are mainly proteins (casein and others) and fatty acids. Expected size range for proteins and fatty acids are respectively from 40nm to 400nm and from 400nm to 10µm.

By observing the size range between 0,5µm-10µm, the displayed information is linked to the fat level, i.e. the fatty acids. This range provides a first assessment of the fat composition of the sample. Lower sizes provide a qualitative information about the casein micelles in the studied sample (resulting of the association of the caseins  $\alpha$ ,  $\beta$ , and  $\kappa$ ).

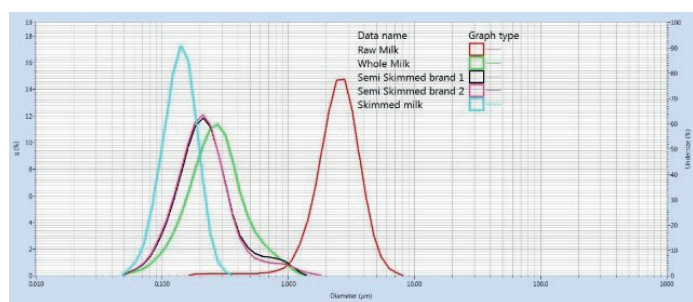


Figure 2. Particle size distributions of different cow milks

Looking at the raw milk curve, the distribution is mainly dominated by native fatty acids as the size distribution presents 99% of particles bigger than 500nm. These large sizes explain the low stability of this kind of milk over the time. The skew observed between 200nm and 1µm can be associated with the casein micelles.

Table 1. Ratio of particles above 500nm in the different milks

| Milk                 | % of particles above 500 nm |
|----------------------|-----------------------------|
| Raw milk             | 98.89                       |
| Whole milk           | 11.29                       |
| Semi-skimmed brand 1 | 8.02                        |
| Semi-skimmed brand 2 | 6.65                        |
| Skimmed milk         | 0                           |

Regarding the commercialized milks, the particle size distributions are a combination of casein micelles (below 500nm) and fat globules (above 500nm). Focusing on the volume fraction above 500nm, a first estimation of the fat level is possible as shown in Table 1.

Looking at the two semi-skimmed milks from different brands, even if slight differences were observed in the particle size analyzes, they were difficult to interpret.

As particle size measurements are not able to provide chemical information, structural identification of the compounds was not possible and the use of a molecular spectroscopic analysis was essential to provide additional informations to the previous assumption.

Therefore, an XploRA™Plus Raman micro-spectrometer was used. The five milks were tested and their Raman spectral fingerprints were acquired. The acquisition took only one second per sample. The spectra are shown in Figure 3.

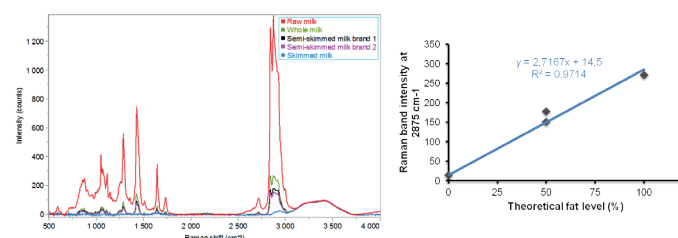


Figure 3. (Left) Raman spectra of different cow milks. (Right) Raman band intensity at 2875 cm<sup>-1</sup> vs. theoretical fat level

These spectra are mixture spectra of fatty acids (all bands below  $3100\text{cm}^{-1}$ ) and water (band in  $3150\text{-}3600\text{cm}^{-1}$  range). By normalizing all spectra by the water band, it becomes easy to quantitatively compare the fat levels of the five studied milks. A linear correlation is obtained and presented in Figure 3 for the commercialized milks. This linear correlation confirms the previous assumption from particle size analyzes about the biggest particles, i.e. fatty acids content.

Concerning the raw milk, a very large contribution from fat molecules was observed. This was also in accordance with the results from particle size analyzes. In terms of quantification, the raw milk was 5-times fatter than the whole milk found in the trade.

Looking at the two semi-skimmed milks from different brands, even if they were announced with the same level of fat, Raman measurements showed that they were slightly different. Raman spectroscopy helped to quantitatively characterize the fat levels with high precision. This precision enables especially to differentiate two different brands simply based on the Raman spectra.

The Aqualog<sup>®</sup> spectrofluorometer has been employed to detect the endogenous fluorescent components in the five milks. Figure 4 shows EEMs for the different types of milk. The Aqualog<sup>®</sup> EEM measuring conditions included an excitation range from 200 to 800nm with a 1nm increment and an emission range of 240-800nm with a 4.65nm CCD bin increment at medium gain and 0.1s integration time.

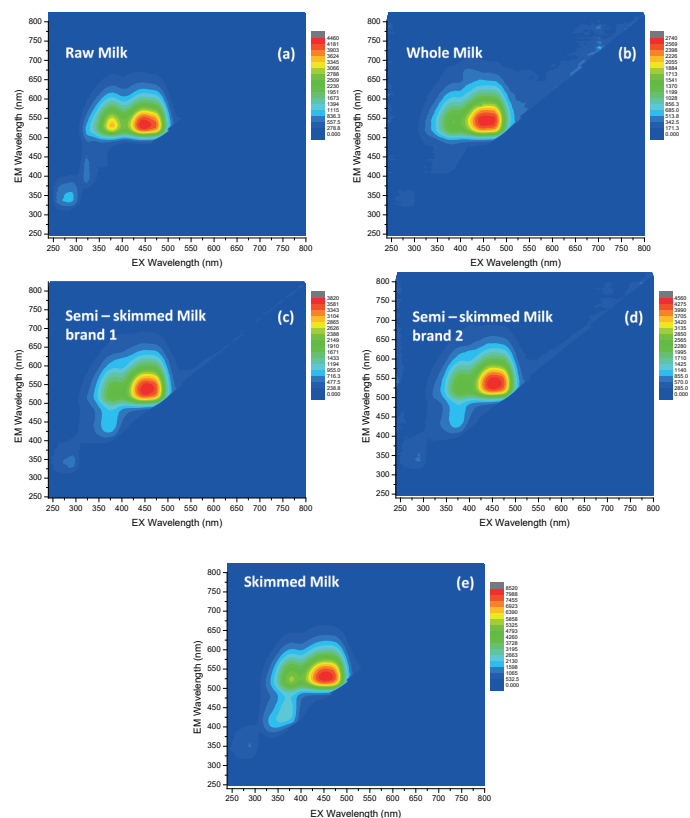


Figure 4. Fluorescence EEMs spectra for Raw milk (a), Whole milk (b), Semi-skimmed milk brand 1 (c), Semi-skimmed milk brand 2 (d) and Skimmed milk (e).

For all the samples several intrinsic fluorophores were detected in the spectral range 300-600nm, including aromatic amino acids and nucleic acids, tryptophan, tyrosine, phenylalanine in proteins, fat-soluble vitamins (retinyl esters) and numerous other compounds. In the 400-530nm spectral region, several emissions overlap and peak assignment was complicated. Raw milk, in particular, showed the most complex structure. Multiple peaks were present, namely 349nm (tryptophan), around 400 and 430nm (fats soluble vitamins like Vitamin A, however, NADH presents a maximum excitation wavelength at about 350nm and may also contribute to the fluorescence observed in this region) and an intense band centered at 524nm attributed to carotenoids, more specifically  $\beta$ -carotene. All these features can be extracted with the Contour Fill Plot Tool which provides a user-specified two-dimensional profile of the EEM (Figure 5).

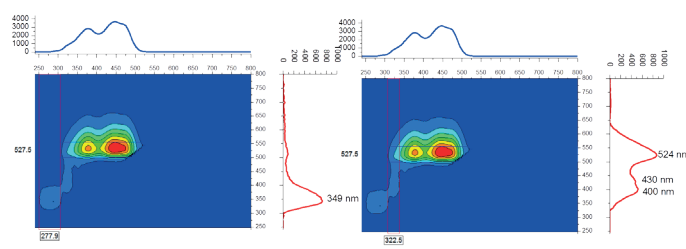


Figure 5. 2D contour plot for Raw milk. (Left) With emission profile for excitation around 278nm. (Right) With emission profile for excitation around 322nm.

## Milk alternatives characterization

As more and more people are subject to cow milk intolerances, many milk alternatives entered the market for few years. Baby powdered milks were one of them since a long time. In this section, we showed how particle size analyzes and optical spectroscopies are powerful analytical tools to characterize that kind of milks and to give information about the milk compositions.

Particle size analyzes of baby powdered milk and soy milk were compared with cow milks results. The corresponding particle size diagrams are shown in Figure 6.

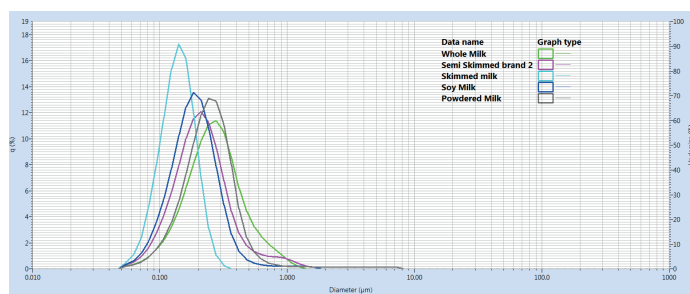
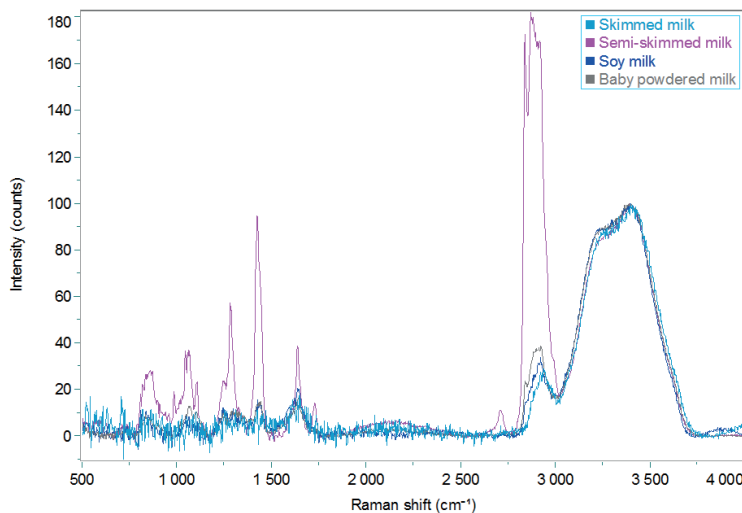


Figure 6. Particle size distributions of different cow milks and milk alternatives.

The baby powdered milk diagram clearly showed that the solution was not enough homogenized as a skew, representing 5% of the total volume was observed from 1 to 10μm.



| Milk alternative   | Raman intensity at 2875 cm <sup>-1</sup> (counts) | Estimated fat level based on linear correlation (%) |
|--------------------|---------------------------------------------------|-----------------------------------------------------|
| Soy milk           | 20                                                | 2                                                   |
| Baby powdered milk | 32                                                | 6                                                   |

Figure 7. (Left) Raman spectra of different cow milks and milk alternatives. (Right) Milk alternative estimated fat levels based on the linear correlation shown in Figure 3.

A first classification of the fat levels was possible based on the fat globules distribution, i.e. the particles in the size range between 0,5µm and 10µm. The soy milk was considered as fatter than skimmed milk as the percentage above 0,5µm was 3% for the soy milk and null for the skimmed milk. For baby milk, semi skimmed milk and soy milk, the fraction between 0,5µm and 2µm was respectively equal to 4,0%, 6,4% and 2,6%. Therefore, baby powdered milk is fatter than soy milk but lighter than semi-skimmed milk.

Once again, even if particle size analysis provides very useful information on particle sizes, it is not a technique for chemical characterization of samples and optical spectroscopies can provide additional information.

In order to classify the milks in terms of fat level precisely, Raman spectra of the different milk alternatives were acquired and compared to cow milks. The spectra are shown in Figure 7.

As shown in the table of Figure 7, fat levels of the two milk alternatives were estimated with the model based on the Raman intensity at 2875 cm<sup>-1</sup> shown in Figure 3. These fat levels were based on easy estimations directly related to the linear correlation previously obtained. Soy and baby powdered milks are a bit fatter than skimmed cow milk, but they contain very low levels of fat - respectively 2% and 6% - compared to the whole milk sample (100%).

Therefore, Raman spectroscopy can be considered as a powerful technique for fat level estimation in milk independently of its origin.

Figure 8 shows EEMs for the milk alternatives. Cow milk and soy milk contain nearly identical amounts of protein and water and fiber is a big plus. In particular, these results confirmed that milk is a rich source of carotenoids. This band was quite intense in both soy and baby milks. The baby powdered milk is also rich in fats soluble vitamins

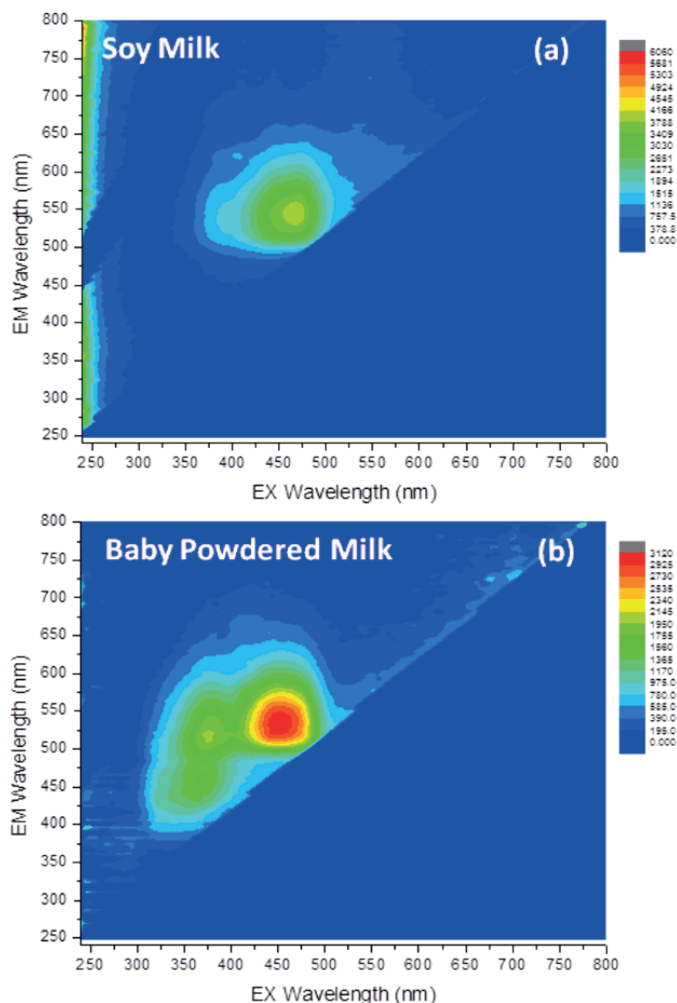


Figure 8. Fluorescence EEMs spectra for Soy milk (a) and Baby Powdered milk (b).



as expected in a processed modified formula for human infants. Figure 9 shows Baby Powdered and Soy Milk profile extractions in the region of interest for vitamins and carotenoids.

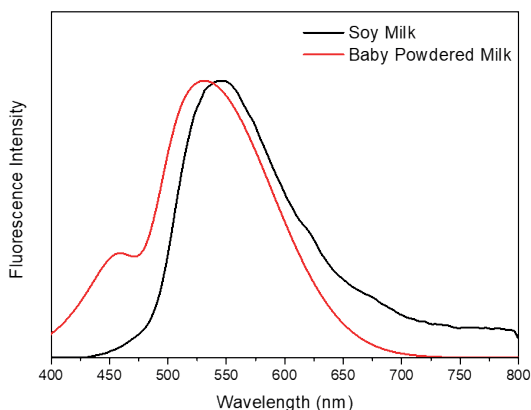


Figure 9. Normalized emission spectra for Soy and Baby Powdered Milk.

## Conclusion:

In this application note, the comparisons between different cow milks and milk alternatives compositions have been realized with success, especially concerning the fatty acids levels and the proteins and vitamins compositions. We have shown that a coupling between particle size analysis, Raman spectroscopy and Fluorescence spectrophotometry enables a large characterization of milk compounds and give complementary information of the studied sample. These techniques are very powerful tools for milk industry quality controls and compound characterization.

Table 2 Key characterization points of the different techniques.

| Technique | Key characterization points                                                                                                                                                                            |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| PSA       | <ul style="list-style-type: none"> <li>Size distribution of components (micelles, fat globules)</li> <li>Ratio between fat globules and micelles</li> <li>First estimation of the fat level</li> </ul> |
| Raman     | <ul style="list-style-type: none"> <li>Compound characterization</li> <li>Fat level determination</li> </ul>                                                                                           |
| Fluo      | <ul style="list-style-type: none"> <li>Identification and relative quantification of endogenous fluorescent vitamins and proteins</li> </ul>                                                           |