Application note

Analysis of Vitamin A Within Cod Liver Oil: Use for Derivatives in UV-Visible Absorption Techniques

Introduction

UV-Visible absorption spectroscopy is a highly useful analytical technique most frequently used for the quantification of analytes within a given solution-phase sample. The method is centered around the ability of molecules to absorb light in the UV-Visible region of the electromagnetic spectrum, which can incite electronic transitions between the ground and excited states. As electronic structure and the associated transition probability are unique to a given molecule, the measured absorption spectrum will provide information specific to the analyte of interest. Through Beer's law (eq. 1), where A is the measured absorbance as a function of wavelength, *c* is the concentration of the analyte, *l* is the pathlength and ε is the extinction coefficient as a function of wavelength, the measured absorption is shown to be linearly proportional to the analyte concentration. This allows for a simple and quick quantitative analysis.

$$A = cl\varepsilon$$

Equation 1.

UV-Visible absorption bands are typically broad due to a variety of factors,[1] and as a result can be complicated to interpret when multiple chromophores are present in solution (Figure 1). While Beer's law (eq. 1) describes the correlation between absorbance and concentration of one chromophore, when multiple chromophores are measured in a single sample the resulting absorption spectrum is additive. Equation 2 describes the relationship between the measured absorbance and the absorption related to each chromophore, where $A_{T,\lambda}$ is the total absorbance and $A_{1,\lambda}$, $A_{2,\lambda}$, and $A_{n,\lambda}$ are the absorbances related to each individual component. For samples with significant spectral overlap, determining the contributions from each chromophore can be difficult or, in the case of principle component analysis, require a certain degree of mathematical fitting and inherent estimation.

$$A_{T,\lambda} = A_{1,\lambda} + A_{2,\lambda} + \cdots + A_{n,\lambda}$$

Equation 2.

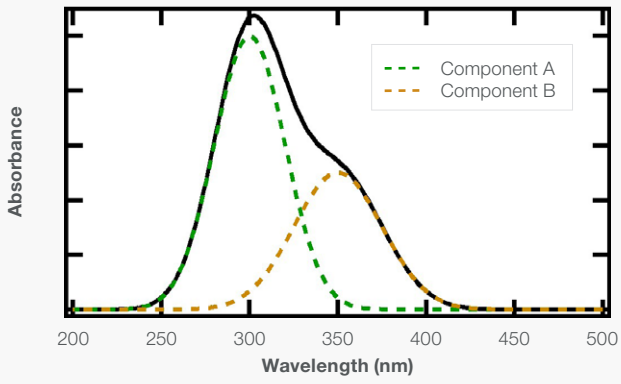

Figure 1. Calculated spectrum of a sample containing two species (black solid line), component A (green dashed line) and component B (orange dashed line).

thermo scientific

To combat these shortcomings, derivative spectroscopy can be employed as a relatively simple method for analyzing the UV-Visible spectra of complex systems. In this method, the n^{th} derivative (1^{st}, 2^{nd}, etc.) is taken of the collected UV-Visible spectrum and graphed as a function of wavelength. These resulting spectra can help better resolve overlapping spectral features[2-5] and are used in a variety of application spaces, such as food science or pharmaceutical analysis.[3,5-8] Though previously obtained through complicated experimental procedures, like wavelength modulation, modern software is able to mathematically calculate the derivative spectra quickly and without need of additional calculations.[9]

The first derivative spectrum represents a plot of the change in the absorbance as a function of wavelength and will include both positive and negative features (Figure 2a). For the analysis of a sample containing a single analyte with no overlapping absorption features, an inflection point will be present at $dA/d\lambda = 0$. This inflection point indicates the location of the absorbance maximum, λ_{max} for the absorbing species present in solution.

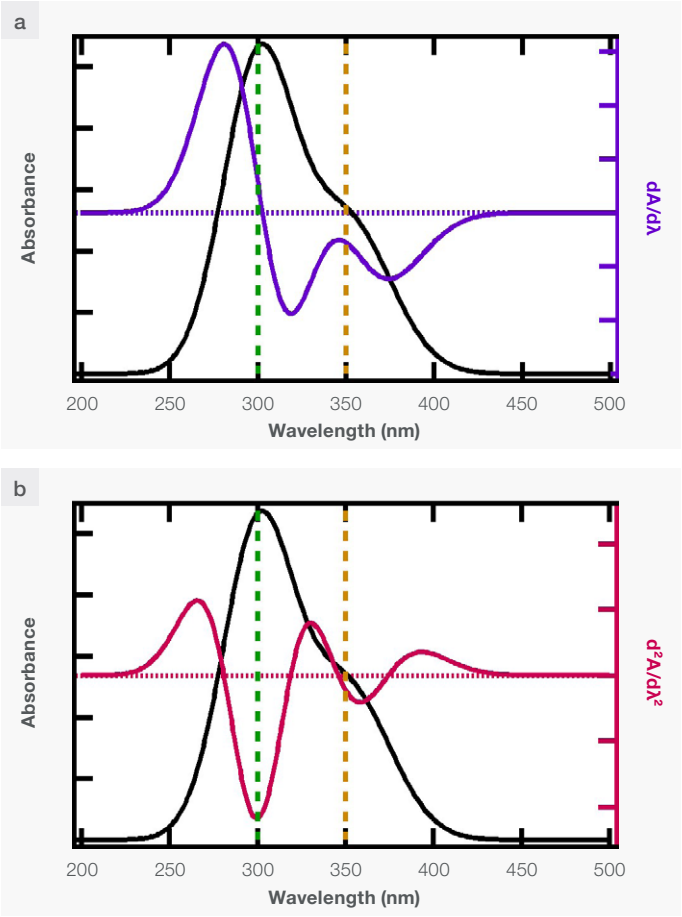

Figure 2. Calculated UV-Visible absorption spectrum (black line) compared to the (a) first and (b) second derivative of the calculated spectrum. Dashed green and orange lines correspond to the location of the maxima for component A and component B, respectively. The dashed horizontal line indicates where the first and second derivatives equal 0.

In complex samples, the first derivative spectrum will be more convoluted and will often not include the inflection point at $dA/d\lambda = 0$. Under these circumstances, determining the exact inflection point can be difficult, especially if the overlapping absorption band is weak. To overcome this issue, the second derivative spectrum can instead be used, as is shown in Figure 2b. Through this analysis, the minima correspond to the location of each respective λ_{max}, which can be easier to visualize comparatively, allowing for a quicker qualitative analysis of complex samples.

It should be noted that though derivative analysis can aid in determining the respective λ_{max} for overlapping absorption bands, this analysis does not completely correct for the effect of overlap. As is shown in Figure 2, the λ_{max} for the weaker band can be slightly shifted with respect to the true λ_{max}. This shift is cause by residual overlapping absorption artifacts and is to be expected in this form of derivative analysis.

While derivative spectroscopy can aid in qualitative analysis, there are methods by which derivative spectra can be quantitatively analyzed as well. These quantitative methods often utilize the first derivative as, according to equation 3, the calculated first derivative is linearly proportional to concentration and thus allows for a simplified analysis approach. [2,5]

$$\frac{dA}{d\lambda} = \frac{d\varepsilon}{d\lambda} cl$$

Equation 3.

Typical absorption methods use the absorption maximum as an analysis wavelength when developing a standard curve; however, this is much more difficult for derivative spectra. As λ_{max} correlates to an inflection point in the first derivative spectrum there should be no change observed in the inflection point as a function of concentration, which means alternative methods of analysis are required.

Analytical methods often cited in the literature include the tangent method (described later in this document), peak-peak, zero-crossing and the ratio method, among others.[2,3,5,10] In these examples, the peak-peak, tangent and zero-crossing methods are all used, though with slightly different calculations, to develop a standard curve relating the calculated quantity to the analyte concentration through a linear function. The ratio method, however, uses the ratio of the negative and positive peaks for the analyte of interest as a point of comparison between a sample and the appropriate standard. Instead of providing a concentration, this analysis serves as a check to determine if there is background interference present in the measured absorption spectrum.

To demonstrate the use of derivative spectroscopy for complex matrices, the vitamin A content within cod liver oil, a common supplement, was analyzed. Cod liver oil is not only known to contain appreciable amounts of vitamin A, also referred to as retinol (Figure 3), but can also contain a variety of other substances, including vitamin D isomers.[13,14] Retinol is known to absorb in the UV-Visible range,[11,12] while at the same time some of the other components present in cod liver oil are also absorptive and include features in the same spectral region as retinol. As a result, the UV-Visible absorption spectrum of cod liver oil is expected to include overlapping absorption features.

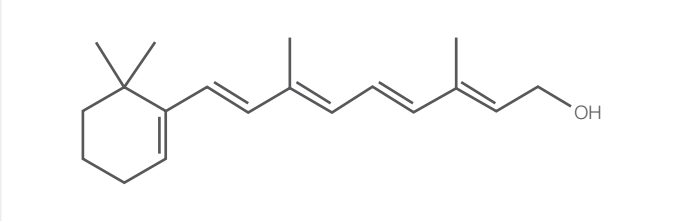

Figure 3. Retinol chemical structure.

Using the Thermo Scientific™ Evolution™ One Plus Spectrophotometer, the absorption spectra of a cod liver oil sample and retinol standard solutions were collected. The resulting data was further processed within the Thermo Scientific™ Insight™ Pro Software to obtain first and second derivative spectra. These resulting spectra were then used to qualitatively and quantitatively analyze the vitamin A content in cod liver oil.

Experimental

Retinol standard samples were made by dissolving 22.4 mg of all-*trans* retinol, hereafter referred to as retinol, in 15 mL absolute ethanol. Using the extinction coefficient as defined in literature (52,770 M^{-1} cm^{-1}),[11] the concentration of the stock solution was found to be 3.69 mM retinol. The prepared retinol sample analyzed in these experiments was 29.5 µM. To prepare the cod liver oil stock solution, 1.0 mL of cod liver oil was diluted in 49.0 mL of absolute ethanol. The sample was then prepared by diluting the cod liver oil stock solution by half.

For the standard curve analysis, five retinol standard solutions of varying concentrations (4.56 – 36.5 USP units/g) were made by diluting the previously prepared stock solution with ethanol. The same cod liver oil sample as described previously was analyzed using the constructed standard curve. The resulting concentration was used to back-calculate the concentration of the original cod liver oil stock solution.

UV-Visible absorption measurements were performed using the Evolution One Plus instrument for all samples. Spectra were collected between 200 nm and 500 nm using a 0.5 s integration time, 1.0 nm bandwidth and 1.0 nm step size. All samples were held in a 1.0 cm quartz cuvette. The first and second derivative spectra of all samples measured were calculated with the Insight Pro Software (Figure 4) using a Savitsky-Golay filter (3 points, second order polynomial).

Figure 4. Derivative analysis within the Insight Pro Software.

Results/Discussion

Figure 5 includes the UV-Visible spectra of both retinol, the vitamin A standard to be used for comparisons, and cod liver oil. The spectrum of retinol includes a peak maximum (λ_{max}) at 325 nm, consistent with literature,[11] as well as a small shoulder feature at 311 nm. Three maxima are observed for the cod liver oil sample (285 nm, 304 nm and 319 nm). It is assumed that the peaks at 319 nm and 304 nm are representative of the vitamin A content present in solution, slightly blue-shifted by ~6 nm due to the presence of a secondary absorption feature centered at 285 nm. This secondary feature is from a separate chromophore present in the cod liver oil. Vitamin D isomers are well known to be present in fish oils and absorb in a region close to this band.[14,15] As a result, it is likely that the band at 285 nm is from a vitamin D isomer, however its exact identification is not the subject of this study.

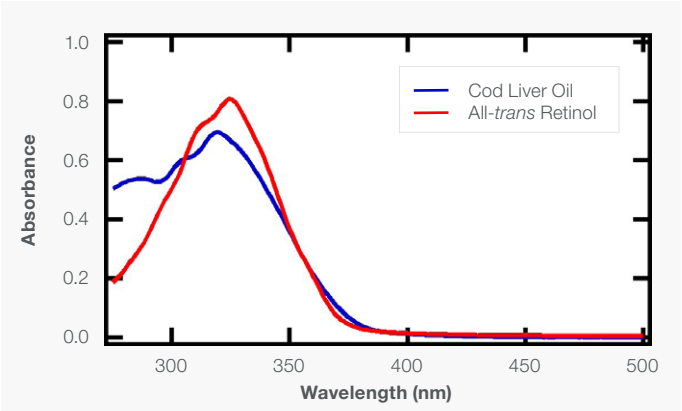

Figure 5. UV-Visible absorption spectrum of retinol (red) and cod liver oil (blue) measured in a 1.0 cm quartz cuvette.

As stated earlier, the presence of an overlapping chromophore can shift the absorption spectrum, making accurate identification difficult. As a second check to more accurately determine the peak maxima locations in cod liver oil, the first and second derivative spectra were calculated for both retinol and cod liver oil (Figure 6). The first derivative spectra for both samples (Figure 6, red lines) match literature[13] and include multiple inflection points. However, the exact location of these inflection points, and therefore the λ_{max}, is difficult to find by eye. Consequently, the second derivative was also calculated for each sample and used to better compare the λ_{max} locations for both retinol and cod liver oil.

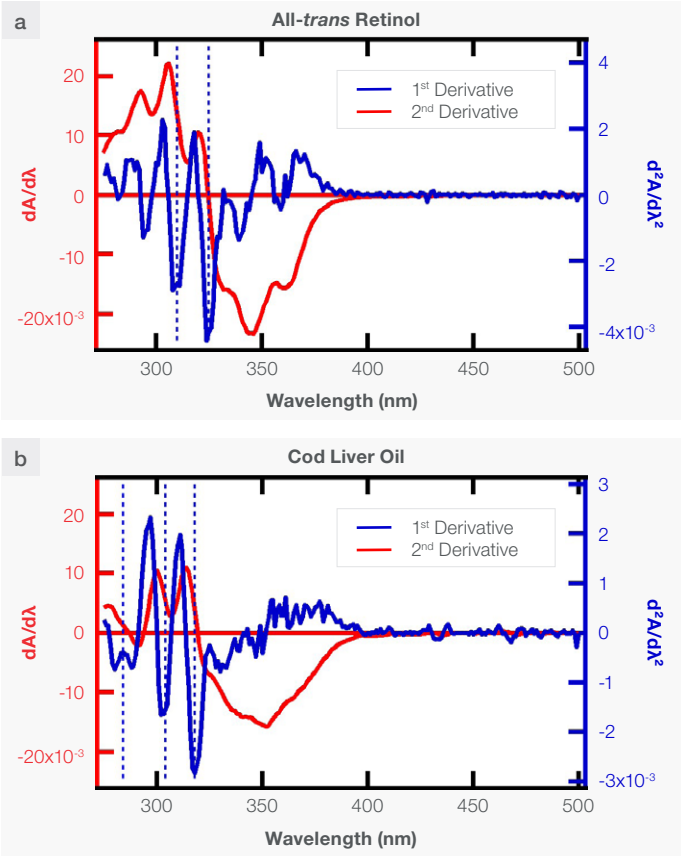

Figure 6. First and second derivative spectra of (a) all-*trans* retinol and (b) cod liver oil. The samples were measured using a 1.0 cm quartz cuvette.

From the second derivative spectrum, two strong minima can be found at 310 nm and 325 nm in the retinol sample, with less intense minima at 295 nm and 360 nm (Figure 6a). The minimum at 328 nm is the characteristic absorption maximum for retinol according to literature.[11] For cod liver oil, three strong minima are observed at 284 nm, 304 nm and 318 nm (Figure 6b). The feature at 284 nm appears to partially overlap with a second minor minimum at 288 nm. The large 284 nm minimum is likely from the second chromophore, assumed to be a vitamin D isomer.

The minima at 304 nm and 318 nm are partially blue-shifted with respect to the strong minima observed in the second derivative spectrum of retinol. However, the difference in wavelength position of these two bands is consistent (14-15 nm), suggesting the blue-shifting is a result of some residual influence from the overlapping chromophore absorption. This is to be expected when comparing an absorption spectrum of a pure standard with an absorption spectrum containing additional absorbing compounds. Overall, these results further support the hypothesis that this spectral feature is related to the vitamin A content within cod liver oil.

The shifted absorption maxima shown in the derivative analysis indicate the contributions from the secondary absorber in the sample are notable. This contribution is likely great enough that a quantitative analysis of the vitamin A content using traditional absorption techniques will be insufficient. Instead, derivative spectroscopy can be used to better quantify the vitamin A concentration and avoid overestimation, an inherent problem when working with multi-component samples.

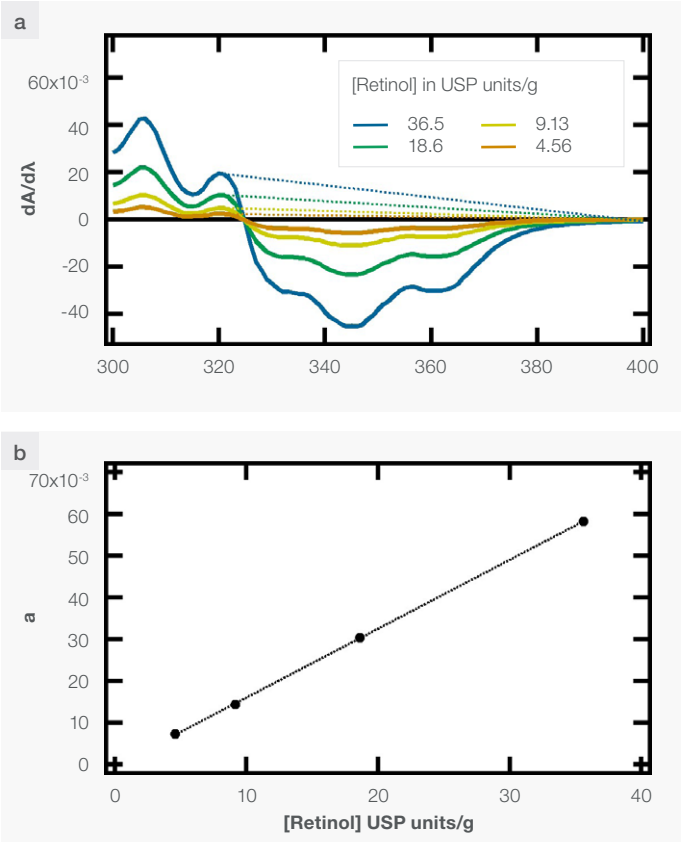

Figure 7. (a) First derivative spectra of all-trans-retinol of varying concentrations. Solid lines are the first derivative spectra and dashed lines are the tangent lines extrapolated using the relative maxima on either side of the minimum of interest. (b) Standard curve for "a" vs retinol concentration. All samples were measured using a 1.0 cm quartz cuvette.

Figure 7a includes the first derivative spectra of five retinol standard samples of varying concentration. As is expected, the amplitude of the positive and negative features increases with increasing concentration of retinol. From this data set, the tangent method was applied to develop a standard curve. This method requires a tangent line connecting the local maxima surrounding the minima of interest, 346 nm for the retinol samples. A line is then drawn from the minimum of interest to the tangent line as shown in Figure 8. This process is repeated for each sample concentration. The magnitude of this line, labeled "a" in Figure 8, is used to develop the standard curve and has been shown to be linear with changes to the analyte concentration.[2]

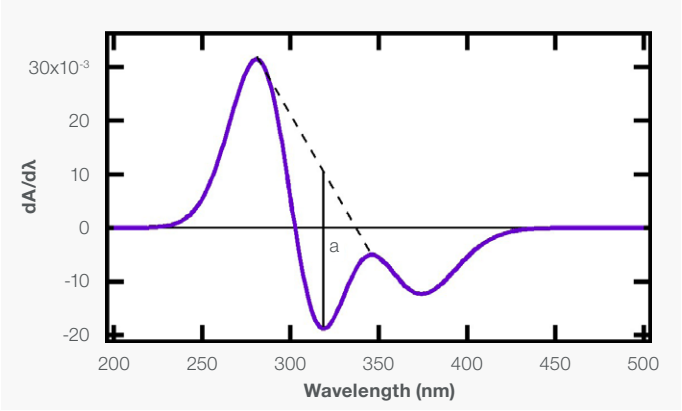

Figure 8. Tangent Method Diagram.

The standard curve for "a" vs retinol concentration is shown in Figure 7b and as anticipated fits well to a line. The same tangent method was used to determine "a" for the cod liver oil samples. The "a" value was found to be 0.023 using the minimum at 351 nm of the first derivative spectrum shown in Figure 6b. Using the constructed standard curve (Figure 7b), the calculated "a" for cod liver oil, and correcting for the dilution factor, the concentration of the vitamin A in the stock cod liver oil sample was found to be 1410 USP units/g. According to the specification sheet for the cod liver oil, the vitamin A concentration was expected to be ≥1383 USP units/g, in good agreement (1.9% difference) with the calculated result using the first derivative.

When the traditional standard curve method was implemented, in which the absorbance at the peak maximum is reported as a function of analyte concentration, the resulting vitamin A concentration in cod liver oil was found to be 1550 USP units/g. This value is 11.7% different from the anticipated result, further highlighting the possible overestimation in complex samples where significant spectral overlap is present.

Conclusions

Through the experiments described herein, UV-Visible derivative spectroscopy was used to determine the vitamin A content in cod liver oil, a complex sample consisting of multiple chromophores. Absorption measurements were collected using the Evolution One Plus Spectrophotometer and easily converted to the respective first and second derivative spectra within the Insight Pro Software. By using derivative spectroscopy, complications from overlapping absorption bands could be avoided, and the vitamin A concentration was found to be in good agreement with the anticipated concentration. Though there are limitations to what can be feasibly resolved with this analysis method, the results described herein demonstrate the utility of derivative spectroscopy and its ease of use.

References

1. Hollas, J. M., *Modern Spectroscopy*; John Wiley and Sons, 2015
2. Talsky, G.; Mayring, L.; Kreuzer, H., High-Resolution, Higher-Order UV/VIS Derivative Spectrophotometry, *Angew. Chem., Int. Ed. Engl.*, **1978**, 17, 785 – 799.
3. Karpińska, J., Derivative Spectrophotometry – Recent Applications and Directions of Developments, *Talanta*, **2004**, 64, 801 – 822.
4. Parmar, A.; Sharma, S., Derivative UV-vis Absorption Spectra as an Invigorated Spectrophotometric Method for Spectral Resolution and Quantitative Analysis: Theoretical Aspects and Analytical Applications: A Review, *Trends Anal. Chem.*, **2016**, 77, 44 – 53.
5. Rojas, F. S.; Ojeda, C. B.; Pavon, J. C., Derivative Ultraviolet-Visible Region Absorption Spectrophotometry and its Analytical Applications, *Talanta*, **1988**, 35, 753 – 761.
6. Atole, D. M.; Rajput, H. H., Ultraviole Spectroscopy and its Pharmaceutical Applications – A Brief Review, *Asian J. Pharma. Clin. Res.*, **2018**, 11, 59 – 66.
7. Habtamu, D.; Belay, A., First Order Derivative Spectra to Determine Caffiene and Chlorogenic Acids in Defective and Nondefective Coffee Beans, *Food Sci. Nutr.*, **2020**, 8, 4757 – 4762.
8. Hassib, S. T.; Asmaa, A. E.-Z.; Marwa, A. F., Validated Stability-Indicating Derivative and Derivative Ratio Methods for the Determination of Some Drugs Used to Alleviate Respiratory Tract Disorders and Their Degradation Products, *Drug Testing and Analysis*, **2011**, 3, 306 – 318.
9. O'haver, T. C., Derivative and Wavelength Modulation Spectrometry, *Anal. Chem.*, **1979**, 51, 91A-99A.
10. Ojeda, C.B., Rojas, F. S., Recent Developments in Derivative Ultraviolet/Visible Absorption Spectrophotometry, *Anal. Chim. Acta*, **2004**, 518, 1 – 24.
11. Barua, A. B.; Furr, H. C., Properties of Retinoids: Structure, Handling, and Preparation, *Molecular Biotechnology*, **1998**, 10, 167 – 182.
12. Furr, H. C., Analysis of Retinoids and Carotenoids: Problems Resolved and Unsolved, *J. Nutr.*, **2004**, 134, 281S – 285S.
13. Wahbi, A. A. M.; Abounassif, M. A.; Alkahtani, H. M., Spectrophotometric Determination of Vitamin A in Oily Capusles Using First Derivative Curves, *Journal of Pharmaceutical and Biomedical Analysis*, **1989**, 7, 39 – 43.
14. Holick, M. F., Vitamin D: A Millenium Perspective, *Journal of Cell Biology*, **2003**, 88, 296 – 307.
15. Orlova, T. N.; Terenetskaya, I. P., Possible Use of Provitamin D3 Photoisomerization for Spectral Dosimetry of Bioactive Antiachitic UV Radiation, *J. Appl. Spectrosc.*, **2009**, 76, 240 – 244.

Learn more at **thermofisher.com/evolution**

thermo scientific