Supplementary Information

Identification and quantification of pigments in plant leaves using thin layer chromatography- Raman spectroscopy (TLC- Raman)

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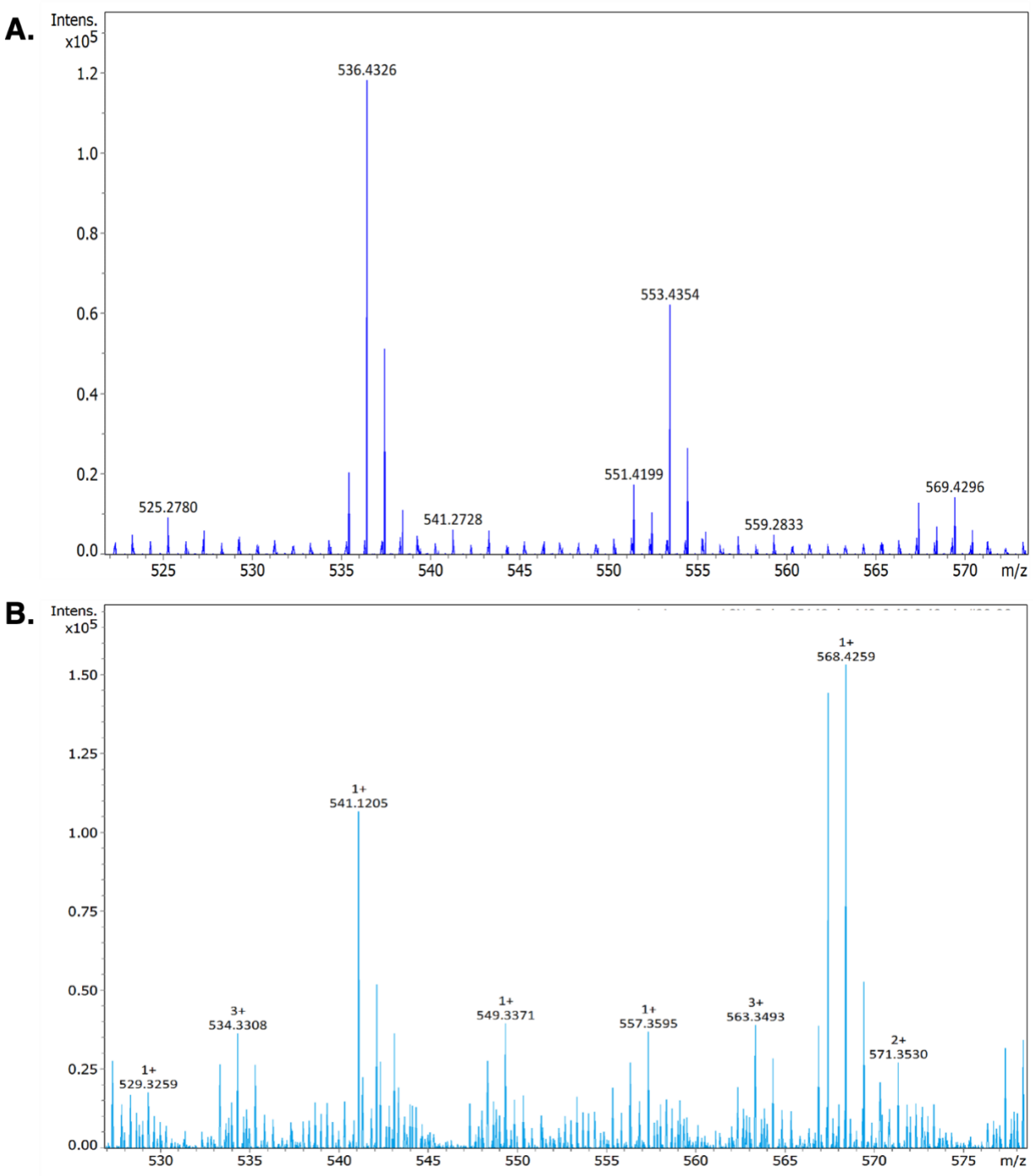
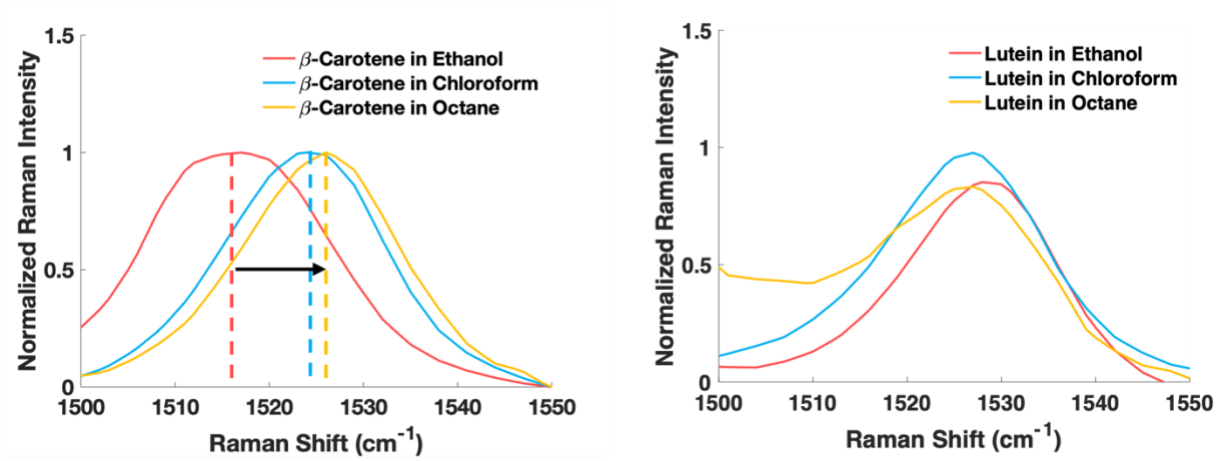


Figure S1. Positive mode ESI LC-MS spectra from **(A)** β-carotene and **(B)** lutein standards used for all experiments. In the top spectrum, the peak at 536.43 m/z corresponds to M^+ where 536.43 is the exact mass of β-carotene. In the bottom spectrum, the peak at 568.43 m/z corresponds to M^+ where 568.43 is the exact mass of lutein.



Solvent	Polarity	Polarizability	C=C Raman shift (cm^{-1})	
			β-Carotene	Lutein
Octane	Nonpolar	High	1526	1527
Chloroform	Moderate	Medium	1524	1527
Ethanol	High	Low	1517	1528

Figure S2. Solvent-related changes in Raman shift (cm^{-1}) of C=C stretch of β-carotene, as compared to relatively constant Raman shift (cm^{-1}) of C=C stretch of lutein, using handheld device.
A Metrohm MiraDS handheld spectrometer equipped with a 785 nm laser and intelligent Universal Attachment was used to collect Raman spectra from solutions of β-carotene and lutein in different solvents. These solvents include octane, chloroform, and ethanol, in order of increasing polarity and decreasing polarizability.[2,3] Samples were dropped onto a gold-coated glass slide, and three spectra from each sample were acquired in orbital raster scanning mode using an acquisition time of 5 s with 37 mW power. The spectra for each sample were averaged, and the height of the peak at ~1520 cm^{-1} was normalized to 1 for comparison of spectra. For β-carotene, the center of the C=C stretching band shifts to the right with decreasing degree of solvent polarity and increasing polarizability. For lutein, the center of the C=C Raman peak remains relatively unchanged based on solvent.

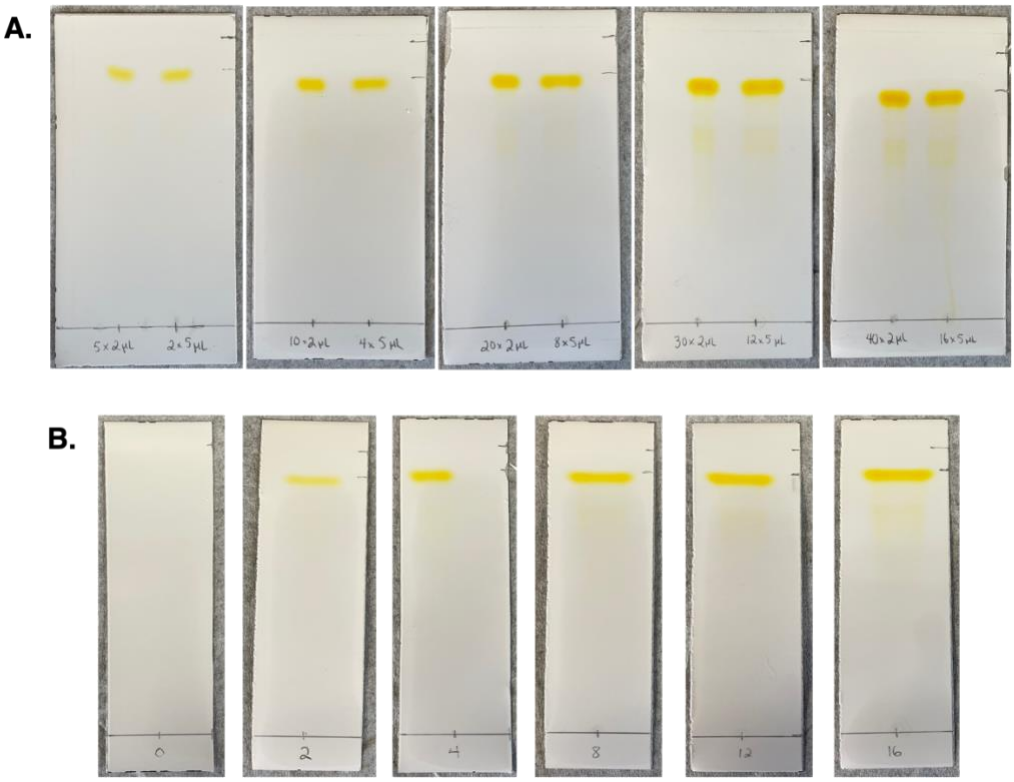


Figure S3. Images of TLC plates from which data was collected for **(A)** TLC-Raman calibration curves for β-carotene using different numbers of both small (2 µL) and large (5 µL) drops and for **(B)** TLC-UV-Vis calibration curve for β-carotene with different numbers of 5 µL drops. R_f = 0.9 for all spots.

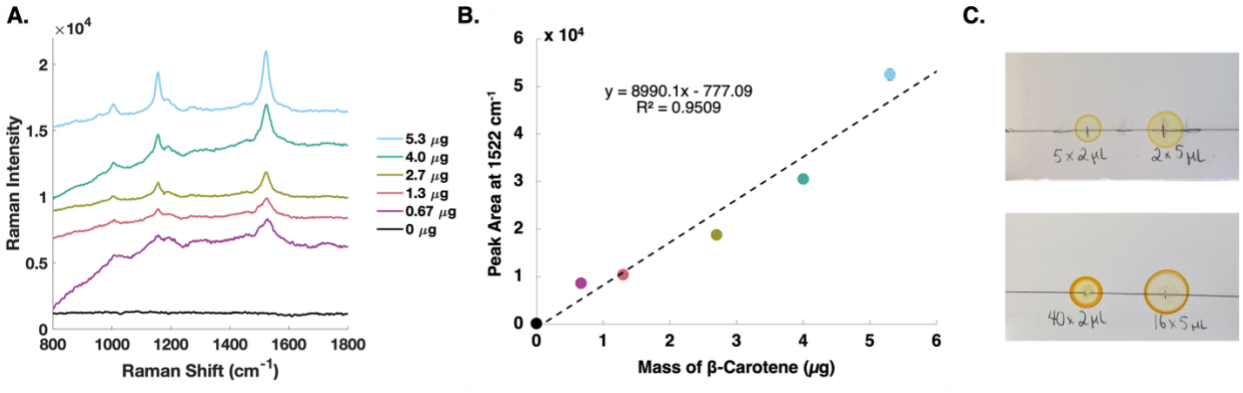


Figure S4. Raman spectroscopy-based calibration curve for β-carotene on TLC plate using larger drops. **(A)** Average Raman spectra from different quantities of β-carotene spotted onto TLC plate using 5 µL drops. Spectra are offset for clarity. **(B)** Plot of average peak area at 1522 cm^{-1} as a function of β-carotene mass loaded onto TLC plate. Standard deviations are included as error bars. **(C)** Comparison of pre-development spot sizes from 2 µL vs. 5 µL drops of sample used to achieve 0.67 µg (top) and 5.3 µg (bottom).
Linear fit (R^2) and sensitivity (m) of this calibration curve are both decreased compared to similar calibration curve created with smaller (2 µL) drops.

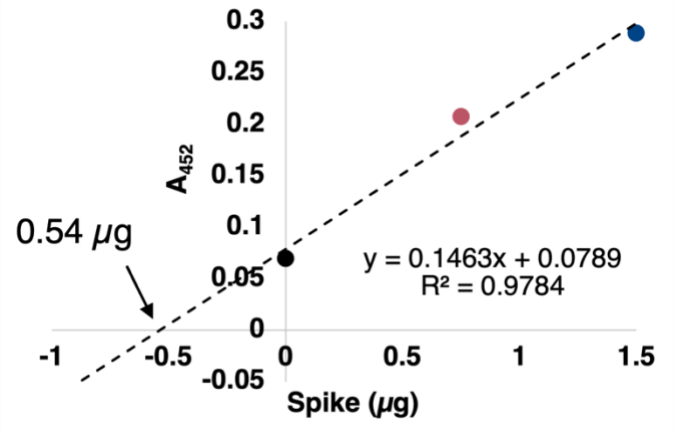


Figure S5. Standard addition curve created using UV-Vis data from leaf extract samples spiked with varying amounts of β-carotene.

Absorbance values at 452 nm are plotted against mass of β-carotene used to spike extract sample. A linear fit is applied to the data, and the fitted line is extrapolated to cross the x-axis. The amount of analyte in the original extract sample is calculated as "-a" where a is the x-intercept.[1] This calculation gives a β-carotene mass of 0.54 ± 0.14 µg, reported with error being equal to standard deviation in concentration (S_x). S_x is calculated by the equation $S_x = \frac{S_y}{m}\sqrt{\frac{1}{N} + \frac{\bar{y}^2}{m^2 S_{xx}}}$, where S_y is standard error in y, m is slope of calibration curve, N is the number of samples used to create the curve, $\bar{y}$ is the average of the calibration values of y_i, and S_{xx} is the sum of squares of deviations of x_i values from $\bar{x}$ with $\bar{x}$ being mean value of x_i. Standard error calculations are shown below for UV-Vis quantification of β-carotene in unspiked extract sample based on standard addition curve.

x_i	y_i
0.00	0.069484
0.75	0.207434
1.50	0.288942

S_y = 0.015135
m = 0.146306
N = 3
$\bar{y}$ = 0.18862
S_{xx} = 1.1250
S_x = 0.139205

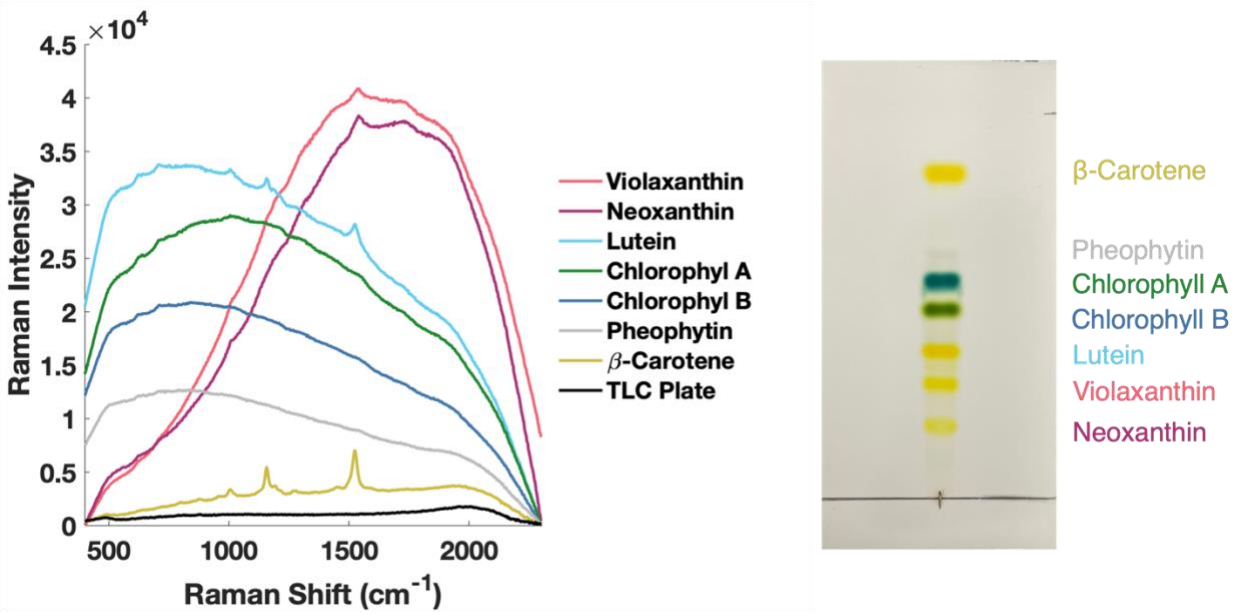


Figure S6. Raman spectra of all pigment spots on TLC plate spotted with spinach leaf extracts.
Organic spinach leaves were purchased from the grocery store and extracted by freezing at -80 ºC, grinding with a mortar and pestle, and soaking in ethanol. A 2 mL aliquot of spinach extract was dried down under N_2(g) and resuspended in 100 µL of acetone. Ten successive 2 µL drops of extract were spotted onto the TLC plate. The plate was developed with mobile phase (petroleum ether:cyclohexane:ethyl acetate:acetone:ethanol (60:16:10:10:6 v/v). Handheld Raman signal at 785 nm was recorded from all observed spots, assigned β-carotene (R_f = 0.9), pheophytin, chlorophyll A, chlorophyll B, lutein (R_f = 0.4), violaxanthin, and neoxanthin (from top to bottom). Power and time used for acquisitions was 23 mW and 10 s for β-carotene, pheophytin, and the control spot on the plate. Acquisition times were reduced to 5 s for lutein, 2 s for neoxanthin, and 1 s for violaxanthin, but power was maintained at 23 mW. Power and time were both reduced to 12 mW and 1 s, respectively, for chlorophyll spots. All pigment spots besides β-carotene exhibit intense fluorescence. The silica plate itself shows minimal background signal.

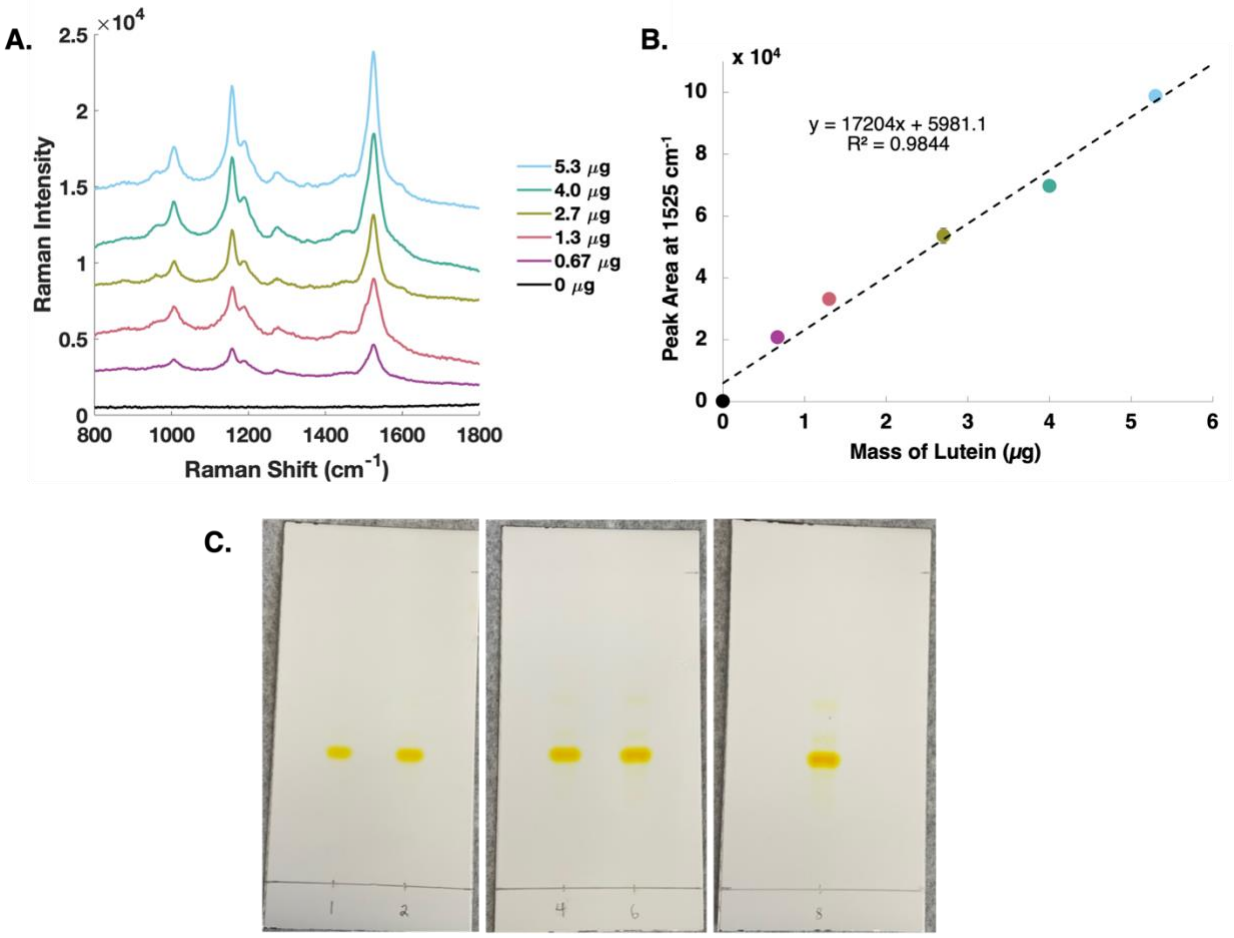


Figure S7. TLC-Raman calibration curve for lutein. **(A)** Average Raman spectra from different amounts of lutein spotted onto TLC plate using 2 µL drops. Spectra are offset for clarity. **(B)** Calibration plot showing average peak area at 1525 cm^{-1} with respect to lutein mass dropped onto TLC plate. Standard deviations are included as error bars. **(C)** Images of TLC plates used to make calibration curve with different numbers of 2 µL drops.
A 0.333 µg/µL stock solution of lutein was prepared in acetone. Various masses of lutein ranging from 0 to 5.3 µg were spotted with a consistent spot size onto TLC plates using different numbers of 2 µL drops of the lutein stock solution. Plates were developed using mobile phase consisting of petroleum ether:cyclohexane:ethyl acetate:acetone:ethanol in a ratio of 60:16:10:10:6 v/v. Photos of each plate were taken, and R_f values were recorded as 0.4 for all spots. Handheld Raman spectra were acquired from each spot in triplicate using a Metrohm MiraDS at 785 nm with a power of 23 mW in raster scanning mode and an acquisition time of 5 s.

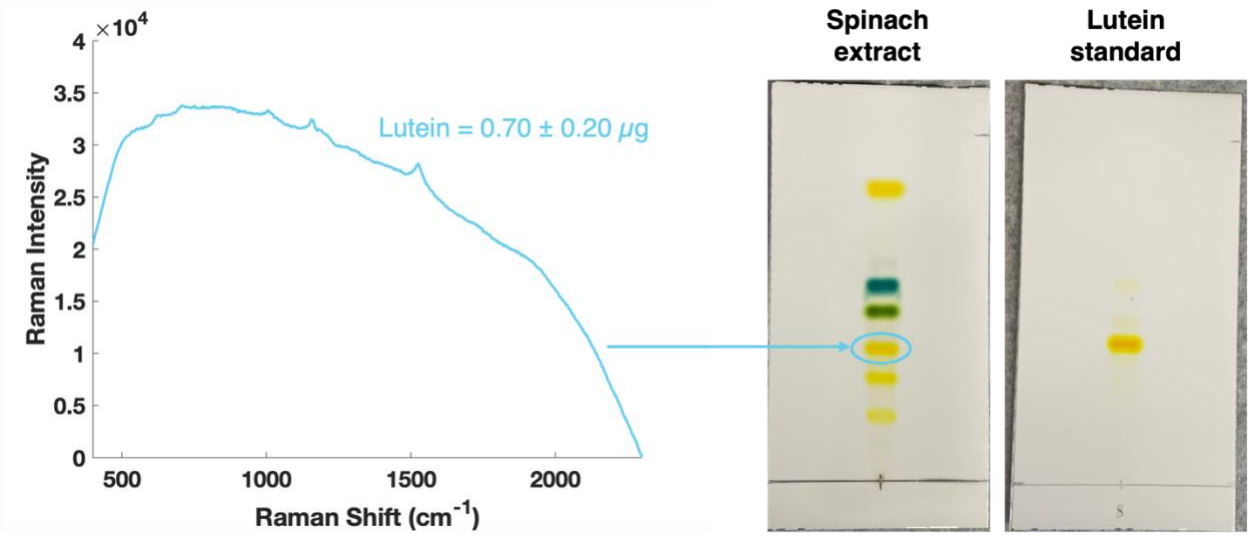


Figure S8. Quantification of lutein in spinach leaf extract.
A TLC plate was spotted with 10- 2 µL drops of spinach leaf extract, then developed using the following mobile phase: petroleum ether:cyclohexane:ethyl acetate:acetone:ethanol (60:16:10:10:6 v/v). After taking a photo of the plate, the R_f value of the lutein spot was recorded as 0.4, which matches the R_f value of the lutein standard as shown. Handheld Raman spectra were acquired from the lutein spot in triplicate using a Metrohm MiraDS at 785 nm with a power of 23 mW in raster scanning mode and an acquisition time of 5 s. Raman peak area at 1522 cm^{-1} was converted to lutein mass using the calibration curve in Figure S7, giving a value of 0.70 ± 0.20 µg of lutein present in 20 µL of the spinach leaf extract.

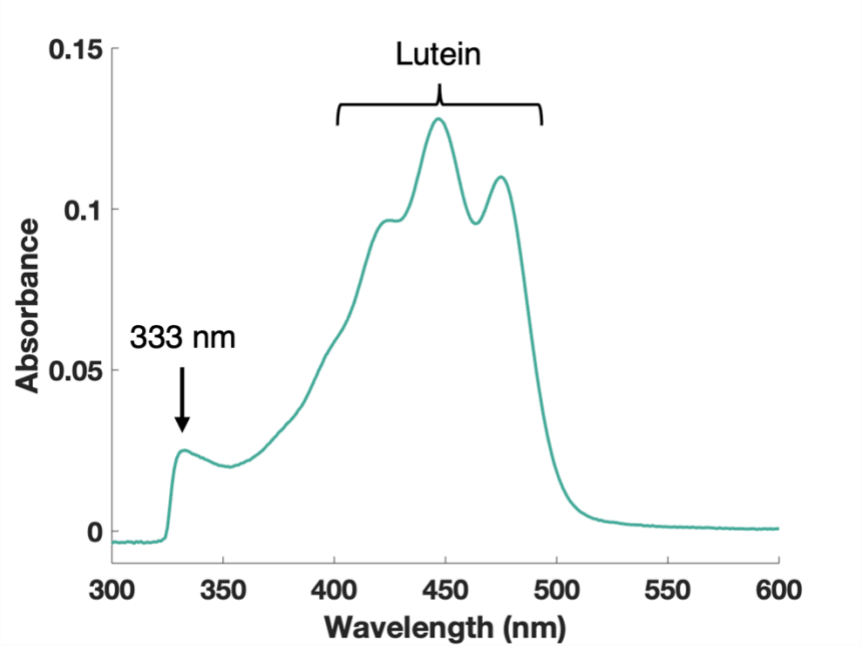


Figure S9: Coinciding molecules present in lutein TLC spot as identified by UV-Vis.
TLC spot of lutein was cut out from the plate, and deposited into a tube containing acetone. The silica was scraped from the plate into the acetone and shaken for 4 min to dissolve. The tube was then centrifuged at 3000 rcf for 1 min to pellet the silica, and the supernatant was transferred into a quartz cuvette. UV-Vis spectra (N = 3) were collected from the supernatant using a Cary 4000 UV-Vis instrument baselined with acetone. Signal at 333 nm in UV-Vis spectra of TLC spot of lutein from spinach leaf extract indicates the presence of additional molecules besides lutein, likely phenolics such as flavonoids, as identified in literature.[4]

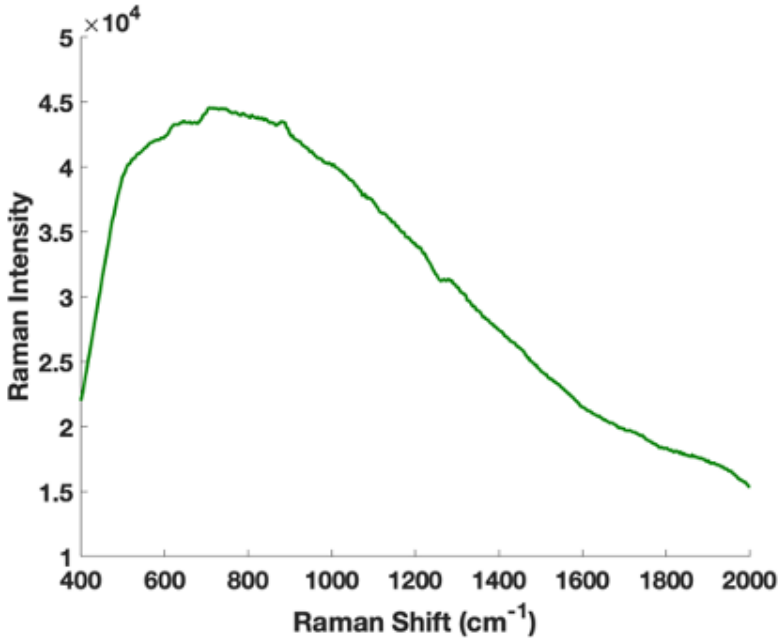


Figure S10. Handheld Raman signal of chlorophyll at 785 nm.
Chlorophyll A was dissolved in ethanol, and solution was dropped onto a gold-coated glass slide. Handheld Raman spectra (N = 3) were acquired using a Metrohm MiraDS at 785 nm in orbital raster scanning mode. Measurements were made using an acquisition time of 2 s with 23 mW of power at the sample. Spectra show intense fluorescence from the chlorophyll.

Calibration curve calculations for detection limit, quantification limit, and standard error in concentration

The limit of detection (LOD) at 95% confidence is calculated by $3s_{bl}/m$ where m is slope of the calibration curve and s_{bl} is standard deviation in blank signal. The limit of quantification (LOQ) at 95% confidence is calculated by $10s_{bl}/m$ where m is slope of the calibration curve and s_{bl} is standard deviation in blank signal.[1]

LODs and LOQs for all calibration curves:

Pigment	Method	s_{bl}	m	LOD (μg)	LOQ (μg)
β-Carotene	**UV-Vis**	0.00068265	0.13605	0.02	0.05
	Raman (2 μL)	189.44	19588	0.03	0.10
	Raman (5 μL)	189.44	8990.1	0.06	0.21
Lutein	**Raman (2 μL)**	189.44	17204	0.03	0.11

The standard error in concentration[1] for each test sample is calculated using $S_x = \frac{S_y}{m}\sqrt{\frac{1}{M}+\frac{1}{N}+\frac{(\bar{y}_c-\bar{y})^2}{m^2 S_{xx}}}$. In this equation S_y is standard error in y, m is slope of calibration curve, M is number of replicate measurements, N is the number of standards used to make the calibration curve, $\bar{y}_c$ is the average response of the unknown, $\bar{y}$ is the average of the calibration values of y_i, and S_{xx} is the sum of squares of deviations of x_i values from $\bar{x}$ with $\bar{x}$ being mean value of x_i. Standard error calculations are shown below for UV-Vis and Raman quantification of β-carotene in unspiked and spiked extract samples based on calibration curves in Figures 3 and 4 of the main text.

Standard errors for calibration curves used for quantification:

β-Carotene UV-Vis:

x_i	y_i
0.0	0.01511145
0.67	0.09312303
1.3	0.21168757
2.7	0.38930144
4.0	0.52716053
5.3	0.75292506

S_y = 0.019004
m = 0.136054
M = 3
N = 6
$\bar{y}$ = 0.331552
S_{xx} = 20.9921

Spike (μg)	$\bar{y}_c$	S_x
0	0.0694837	0.114907
0.75	0.207434	0.102610
1.50	0.288942	0.099229

β-Carotene Raman (2 μL):

x_i	y_i
0.0	138.6763
0.67	7072.652
1.3	18054.95
2.7	43156.83
4.0	82876.19
5.3	96653.91

S_y = 5479.641
m = 19587.68
M = 3
N = 6
$\bar{y}$ = 41325.53
S_{xx} = 20.9921

Spike (μg)	$\bar{y}_c$	S_x
0	6075.611	0.226282
0.75	20090.32	0.208594
1.50	34536.85	0.198941

Lutein Raman (2 μL):

x_i	y_i
0.0	138.6763
0.67	20840.56
1.3	33118.31
2.7	53619.12
4.0	69695.55
5.3	98820.21

S_y = 4390.999
m = 17204.43
M = 3
N = 6
$\bar{y}$ = 46038.74
S_{xx} = 20.9921

For spinach leaf extract:
$\bar{y}_c$ = 18037.33
S_x = 0.201965

References

1. D. A. Skoog, F. J. Holler and S. R. Crouch, in *Principles of Instrumental Analysis*, Cengage Learning, Boston, MA, Seventh., 2018, pp. 11, 13–14, 18, 904.

2. Experimental Polarizabilities. https://cccbdb.nist.gov/pollistx.asp (accessed 2023-11-16).

3. L. R. Snyder, *Journal of Chromatography A*, 1974, **92**, 223–230.

4. I. Spiridon, R. Bodirlau and C.-A. Teaca, *Open Life Sciences*, 2011, **6**, 388–396.