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Quantitative Raman Spectroscopy for the Analysis of Carrot Bioactives

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ABSTRACT: Rapid quantitative near-infrared Fourier transform Raman analyses of the key phytonutrients in carrots, polyacetylenes and carotenoids, are reported here for the first time. Solvent extracts of 31 carrot lines were analyzed for these phytonutrients by conventional methods, polyacetylenes by GC-FID and carotenoids by visible spectrophotometry. Carotenoid concentrations were 0−5586 μg g^{-1} dry weight (DW). Polyacetylene concentrations were 74−4846 μg g^{-1} DW, highest in wild carrots. The polyacetylenes were falcarinol, 6−1237 μg g^{-1} DW; falcarindiol, 42−3475 μg g^{-1} DW; and falcarindiol 3-acetate, 27−649 μg g^{-1} DW. Strong Raman bands for carotenoids gave good correlation to results by visible spectrophotometry. A chemometric model capable of quantitating carotenoids from Raman data was developed. A classification model for rapidly distinguishing carrots with high and low polyacetylene (limit of detection = 1400 μg g^{-1}) concentrations based on Raman spectral intensity in the region of 2250 cm^{-1} was produced.

KEYWORDS: *Daucus, carrot, Raman, polyacetylenes, carotenoids, spectroscopy, chemometrics*

INTRODUCTION

Two important classes of natural products of carrots (*Daucus carota* L., Apiaceae) are carotenoids and polyacetylenes. These compounds demonstrate bioactivity in humans, which is believed to result in health benefits associated with dietary fruits and vegetables.[1−3] In recent years the bioactivity, bioavailability, structural diversity, and localization/concentration of polyacetylenes in carrots has come under increasing scrutiny, with particular focus on their function as health-promoting phytonutrients.[4−6] Three principal polyacetylenes are found in carrots: falcarinol (FOH), **1**; falcarindiol (FDOH), **2**; and falcarindiol 3-acetate (FDOH3Ac), **3** (Figure 1). Polyacetylenes have shown activity against cancer cells in vitro and against preneoplastic lesions in rat colon in vivo.[7,8] FOH, **1**, plasma concentrations have been shown to increase within an hour of consumption of carrot juice, demonstrating the bioavailability of these compounds.[6] Dietary carotenoids are bioavailable antioxidants believed to play a role in mitigating oxidative stress and hence in combating chronic diseases.[9,10]

The concentrations of both of these classes of compounds have been shown to vary widely across carrot varieties.[11−13] This variability in phytonutrient concentration has been related to many factors including carrot variety, root size, growth conditions, and, in the case of polyacetylenes, root damage and postharvest storage conditions.[13−15] As such, a wide range of variation in the concentration of these phytonutrients can be expected from different harvests in different areas, even when the same cultivar is being used. This is exemplified for polyacetylenes in a study by Yates et al., in which polyacetyl-ene content was shown to vary by as much as ±40% in carrots of the same variety grown at different locations.[16]

Current methods to quantitate carotenoids and polyacetylenes are time-consuming and require destructive extractions.[17−19] A fast analytical method capable of simultaneously quantifying polyacetylenes and carotenoids in carrots could be useful to carrot breeders selecting for these phytonutrients.

Raman spectroscopy is starting to be used in the analysis of plant material.[20] The technique is nondestructive and, with the development of hand-held instruments, can be used to provide rapid in-field analysis. The difficulties of fluorescence from sample matrices may be mitigated by using near-IR excitation wavelengths such as 1064 nm.[21,22] The complexity of vibrational spectral data and the convolution of useful sample data with background noise make robust data analysis very important. Chemometrics is a powerful analysis tool for such data, providing the ability to quantify specific chemical species within a complex matrix.[23−27]

Raman spectral analysis in conjunction with chemometrics has been applied to the quantitative analysis of plant matrix constituents with varying success. Chemometric models have been developed capable of the semiquantitative determination of harpagoside concentrations in Devil's Claw,[26] aspalathin content of green rooibos tea,[28] and unsaturated acyclic sulfur

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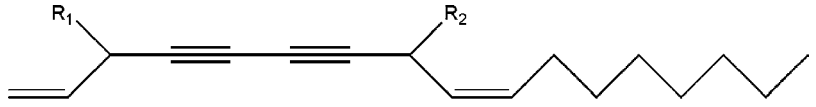

(1) Falcarinol $R_1 = OH$ $R_2 = H$
(2) Falcarindiol $R_1 = OH$ $R_2 = OH$
(3) Falcarindiol 3-acetate $R_1 = OAc$ $R_2 = OH$

Figure 1. Structures of carrot polyacetylenes.

compounds in garlic.[29] Qualitative and quantitative chemometric analyses of natural oils have been performed in diverse biological matrices including rapeseed and salmon.[24,25,30,31] Raman spectroscopy has also been used to show localization of carotenoids and polyacetylenes, as well as other components in the plant matrix, in cross sections of carrot root.[32−34]

This study examined the potential of Raman spectroscopy for quantitative analyses of phytonutrients in carrots. To this end, carrots were analyzed using three different Raman excitation wavelengths (1064, 785, and 830 nm) and both FT and dispersive spectrometer arrangements. Spectra were subjected to multivariate analyses, which, in conjunction with GC-FID and visible spectrophotometry results, were used to produce chemometric models for the quantitation of carrot phytochemicals.

MATERIALS AND METHODS

Plant Material. Thirty-one carrot lines, selected to encompass a wide genetic variability from wild varieties to commercial cultivars, were supplied by Vilmorin SA. Carrots were grown from seeds at Kairanga on the Manawatu Plains, North Island, New Zealand (40° S, 175° E) in Kairanga silt loam soil. All 31 lines were planted on February 1, 2011, and thinned to leave approximately 30 mm between plants on March 31. Three replicates of each carrot line were produced by randomly allocating plots for each line in three separate blocks. Blocks were next to one another and showed no obvious variation. A single artificial irrigation was applied on February 22 to help establish the plants. Carrot fly was controlled by applying diazinon prills (Yates soil insect killer, 20 g ai kg^{-1}) at a rate of 1 g m^{-1} of row at sowing before closing the drills. A second treatment was applied on March 15, 2011. Slugs were controlled by broadcasting metaldehyde prills at emergence on February 15 and again on February 28. Weeds were controlled by pre-emergence application of linuron at 3 kg ha^{-1} on February 3. Carrots were harvested by hand on June 17, cleaned of soil, and held in plastic bags at 2 °C until June 20−21, when they were sampled for freeze-drying. From 8 carrots a 20 mm slice was cut from midheight, diced, and mixed. A portion of this dice was frozen in liquid nitrogen prior to freeze-drying and storage at −20 °C until analysis. Some lines, especially the wild varieties, had small roots, and more than eight carrots were therefore sampled.

Isolation, Purification, and Identification of Polyacetylene Standards. Commercial carrots (850 g) were lyophilized, and the resulting dry matter (95.1 g) was extracted with dichloromethane (DCM, 2 × 950 mL). The combined extracts were dried (1.25 g) and fractionated on a silica column with a DCM/EtOAc gradient elution. Fractions were analyzed and combined on the basis of GC-MS, which showed the elution order to be FOH, **1**; FDOH3Ac, **3**; and FDOH, **2**. The combined fractions rich in **1**, **3**, or **2** were purified by HPLC using an Agilent 1260 Infinity and Phenomenex Luna (5 μm) C18 column (10 × 250 mm). Gradient elution from 70 to 88% methanol in H_2O was applied over 30 min followed by 100% methanol for 5 min and a 5 min re-equilibration. The flow rate was 4.5 mL min^{-1}, the injection volume was 200 μL, and detection was at 210 nm. All three polyacetylenes were pale yellow gums with purities of >99% by GC-FID and ^{1}H NMR data that matched those of Czepa and Hofmann.[17]

Quantitation of Polyacetylenes by GC-FID. Freeze-dried carrot powder (0.1 g) was accurately weighed and extracted into EtOAc (1 mL) with sonication for 15 min. Fifty microliters of an eicosane solution (1 mg mL^{-1} EtOAc) was added to each sample as an internal standard. Samples were centrifuged at 5000 rpm for 5 min, and an aliquot of supernatant was filtered (0.45 μm) into GC vials for analysis. Three agronomic replicates of each carrot line were analyzed with an injection volume of 1 μL. Polyacetylenes were quantitated using seven-point calibration curves ranging from 0 to 500 μg mL^{-1} FOH, **1**; FDOH, **2**; and FDOH3Ac, **3**. An Agilent 7890A gas chromatograph with a CTC Analytics PAL system autosampler with FID detector (350 °C) equipped with a 30 m × 0.25 mm i.d. HP-5-ms column with 0.25 μm film was used for analysis. The injector (260 °C) had a split ratio of 20:1. The carrier gas was H_2 at a flow rate of 1.5 mL min^{-1}. The oven was heated from 150 to 300 °C at 10 °C min^{-1} and had a hold time of 5 min. This method was based on that used by Metzger and Barnes.[11]

Quantitation of Carotenoids by Visible Spectrophotometry. The carrot extracts from above were diluted 20-fold with EtOAc and analyzed by UV−vis in the range of 400−550 nm. Spectral intensity at 452 nm was measured for each sample and quantitated against a seven-point β-carotene (Sigma-Aldrich, ≥93%) calibration curve in the range of 0−1550 μg mL^{-1} to give an estimate of total carotenoid content. This method was adapted from the current USP-NF method for quantitation of carotenoids.[35]

Raman Analyses. *Sample Presentation.* Freeze-dried carrot powders (∼50 mg) were packed into aluminum divots prior to analysis (diameter = 3 mm, depth = 5 mm). When possible, lasers were focused to optimize the check signal prior to analysis. Three agronomic replicates of each carrot line were analyzed.

Raman Spectroscopy Using 1064 nm Excitation. Raman spectra were recorded using a Bruker FT-Raman spectrometer (model FRA 106/5) equipped with a Nd:YAG laser emitting at 1064 nm and a germanium detector cooled with liquid N_2. Spectra were acquired at a laser power of 120 mW, from 0 to 3500 cm^{-1} Stokes shift from the incident laser frequency and with a spectral resolution of 4 cm^{-1}. Acquired spectra were the average of 100 scans with a laser diameter of approximately 0.3 mm. The total acquisition time was approximately 3 min.

Raman Spectroscopy Using 830 nm Excitation. Raman spectra were recorded using a BWTEK portable iRaman spectrometer equipped with a direct frequency diode emitting at 830.1 nm and TE cooled linear array CCD detector. Spectra were collected using a fiber optic interface cable. The maximum instrument power (271 mW) was used with spectra acquired from 199 to 2302 cm^{-1}. The final acquired spectra were an average of 16 scans with each scan consisting of 8 × 1 s exposures. The total acquisition time was approximately 4 min.

Raman Spectroscopy Using 785 nm Excitation. Excitation was at 784.9 nm using an Ondax laser diode (model Surelock LM series) equipped with two Optigrate BragGrate bandpass filters to suppress amplified spontaneous emission (ASE). Light scattered from the sample was collected by a lens (f/2.3) in a backscattering geometry before being passed through three Optigrate BragGrate notch filters and finally being focused onto a fiber optic coupling (Princeton Instruments fiber coupling model FC-446-021-U, filter chamber model ARC-446-070). Raman spectra were recorded using a Pixis 100 BR eXcelon CCD detector. The acquired spectra were an average of 16 scans with each scan consisting of 8 × 1 s exposures. The total acquisition time was approximately 4 min.

Multivariate Analysis of FT-Raman Spectra. Analysis was performed using the CAMO multivariate data analysis software The Unscrambler. Spectral regions showing variability due to optical artifacts (0−200 cm^{-1}) and background noise (200−400, 1700−2200, 2300−2800, and 3050−3500 cm^{-1}) were omitted from consideration prior to any preprocessing. Smoothing and baseline corrections were not deemed necessary due to the high quality of the spectra and because attempts to do so resulted in an unacceptable loss in spectral data. Standard normal variate (SNV) and multiplicative scatter correction (MSC) transformations were performed to center and scale spectra. Potential outliers, as identified by The Unscrambler software, were investigated using Hotelling (T^2), leverage, and residual values. After investigation, statistically conspicuous spectra were either kept or omitted from models on the basis of visual assessment. Quantitative models were designed by correlating spectral data to absolute polyacetylene and carotenoid values using multiple linear regression (MLR), principal component regression (PCR), and partial least-squares regression (PLS-R). Data for each model are presented in Table 3.

Dose Response and Detection Limits. Raman dose response curves were produced for β-carotene and polyacetylenes by plotting the integral of key Raman bands against the concentration of the relevant phytonutrient dispersed in cellulose powder. For polyacetylenes, an r^2 value of 0.995 was obtained by plotting the integral of the peak at 2250 cm^{-1} against known polyacetylene concentrations in the range of 1388−10984 $\mu g\ g^{-1}$ (Figure 2). For β-carotene, an r^2 value of 0.988 was obtained by plotting the integral of the peak at 1520 cm^{-1} against known β-carotene concentrations in the range of 42−10031 $\mu g\ g^{-1}$ (Figure 3). Detection limits were 42 and 1400 $\mu g\ g^{-1}$ for carotenoids and polyacetylenes, respectively.

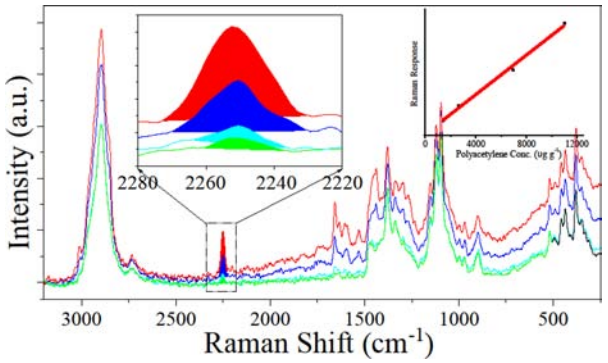

Figure 2. 1064 nm FT-Raman spectra of cellulose powder spiked with parsnip extract (1388−10984 $\mu g\ g^{-1}$) with inset response curve.

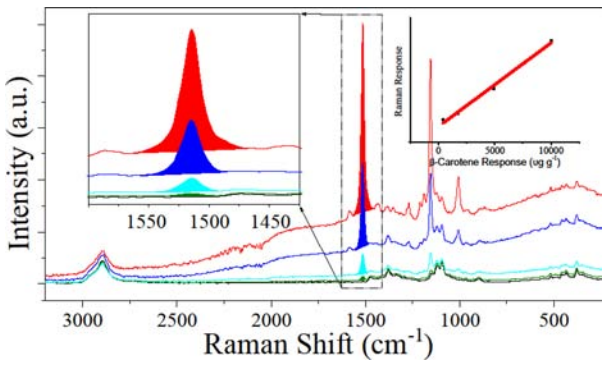

Figure 3. 1064 nm FT-Raman spectra of cellulose powder spiked with β-carotene (42−10031 $\mu g\ g^{-1}$) with inset response curve.

RESULTS AND DISCUSSION

Quantification of Polyacetylenes by GC-FID. Analysis of polyacetylenes by GC-FID was based on the method of Metzger and Barnes.[11] Total polyacetylene concentrations varied greatly (Table 1): highest in wild carrot lines, up to 4846 $\mu g\ g^{-1}$ DW; and lowest in a pink intermediate variety, at just 74 $\mu g\ g^{-1}$ DW. FDOH, **2**, was the most highly concentrated polyacetylene in 23 lines, whereas FDOH3Ac, **3**, was present in the lowest concentrations in 27 of the 31 lines analyzed (Table 1). Individual measurements showed a wide variability across agronomic replicates from certain lines. Because analytical replicates of polyacetylene concentrations in a check sample varied by <10% (n = 8, Table 1), this variability was most likely representative of real variation between the agronomic replicates.

Polyacetylene concentrations have previously been shown to vary with genotype, root size, growth location, harvest year, storage time, and processing.[11,13,16−18,36,37] The range of total polyacetylene concentrations found by our study was in line with previous results, but the relative abundance of individual polyacetylenes differed from some studies. Metzger and Barnes found that the most highly concentrated polyacetylene in 11 of 16 carrot lines was FDOH3Ac, **3**.[11] By contrast, either FDOH or FOH, **1**, was the main polyacetylene in all but 1 of the 31 lines we studied (Table 1). In general, the carrot varieties analyzed by Metzger and Barnes[11] had higher total polyacetylene contents than the carrots in our study. This may be due to the comparatively high FDOH3Ac, **3**, content reported in the previous study. Our fractionation of a bulk carrot extract, to isolate polyacetylene standards for calibration, yielded FDOH3Ac, **3**, in considerably lower quantities than the other two polyacetylenes. This was a direct reflection of the relative peak sizes by GC-FID and therefore supports FDOH3Ac, **3**, being present at the lowest concentration in most of the carrots that we analyzed.

Pferschy-Wenzig et al. found FOH, **1**, concentrations in 27 carrot genotypes in the range of 46−257 $\mu g\ g^{-1}$ DW,[18] within the range found by us (Table 1). Another study on four carrot varieties showed FOH, **1**, concentrations ranging from 213 to 585 $\mu g\ g^{-1}$ DW,[36] which again falls in line with results from our study. Yates et al.[16] showed total polyacetylenes to range from 335 to 1958 $\mu g\ g^{-1}$ DW (assuming 90% water content) but did not quantitate FDOH3Ac, **3**. FDOH, **2**, was the most highly concentrated polyacetylene in all varieties analyzed in that study. [16]

Quantification of Carotenoids by Visible Spectrophotometry. Carotenoid concentrations varied as expected from the carrots' colors, with the deepest orange carrots having the highest carotenoid concentrations (Table 2). Fourteen of the carrot lines examined were orange of various intensities and had total carotenoid concentrations from 2894 to 4908 $\mu g\ g^{-1}$ DW. The two notably deep orange carrots had carotenoid concentrations of >5000 $\mu g\ g^{-1}$ DW, whereas extracts of the four wild, two albino, and three pink lines had carotenoid concentrations below the detection limit at 452 nm.[35] Purple carrots showed low concentrations of carotenoids, 326−728 $\mu g\ g^{-1}$ DW. One unusual line had a purple outside and an orange inside and a carotenoid concentration of 3008 $\mu g\ g^{-1}$ DW.

The carotenoid concentrations of carrots in this study compared well with those reported in other studies (assuming 90% water content).[11,12,38−40] The total carotenoid content of carrots analyzed by Metzger and Barnes[11] by HPLC had the

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Table 1. Polyacetylene Concentrations across 31 Carrot Lines (Micrograms per Gram DW; Mean of Three Agronomic Replicates ± Standard Deviation) by GC-FID and FT-Raman

					total polyacetylenes	
line	type	FOH	FDOH	FDOH3Ac	GC-FID	FT-Raman[a]
1	Nantes	188 ± 20	238 ± 91	154 ± 38	579 ± 141	397
2	intermediate	68 ± 54	183 ± 103	55 ± 24	306 ± 83	637
3	Imperator	434 ± 104	345 ± 97	87 ± 13	865 ± 209	465
4	Nantes	191 ± 35	241 ± 44	108 ± 16	540 ± 87	405
5	intermediate	6 ± 2	42 ± 16	27 ± 12	74 ± 29	1366
6	Nantes	336 ± 52	271 ± 63	85 ± 5	692 ± 107	346
7	intermediate	197 ± 19	248 ± 56	154 ± 19	599 ± 91	383
8	Imperator	266 ± 30	200 ± 78	71 ± 16	538 ± 118	350
9	Nantes/Kuroda	334 ± 161	222 ± 83	68 ± 26	625 ± 270	469
10	intermediate	499 ± 108	366 ± 12	139 ± 19	1004 ± 92	562
11	Old French	495 ± 119	528 ± 108	264 ± 114	1287 ± 294	394
12	Imperator	316 ± 91	387 ± 32	152 ± 41	856 ± 156	366
13	Nantes	368 ± 36	240 ± 90	123 ± 25	731 ± 149	290
14	intermediate	330 ± 44	468 ± 115	47 ± 15	845 ± 166	589
15	Imperator	274 ± 72	401 ± 42	98 ± 19	773 ± 68	533
16	Kuroda	305 ± 51	400 ± 13	90 ± 15	794 ± 44	538
17	intermediate	588 ± 92	663 ± 134	147 ± 40	1397 ± 79	998
18	wild	597 ± 63	2587 ± 379	442 ± 71	3626 ± 508	1762
19	wild	1237 ± 62	1848 ± 321	282 ± 30	3367 ± 311	1409
20	Nantes	112 ± 82	497 ± 499	107 ± 153	716 ± 730	1071
21	intermediate	338 ± 45	761 ± 226	246 ± 72	1345 ± 336	925
22	Kuroda	195 ± 37	300 ± 78	123 ± 45	618 ± 157	598
23	Flakkee	242 ± 71	557 ± 147	254 ± 122	1054 ± 214	367
24	wild	648 ± 78	3221 ± 920	518 ± 128	4386 ± 1025	1750
25	Intermediate	198 ± 26	331 ± 27	118 ± 26	647 ± 75	357
26	wild	748 ± 201	3475 ± 280	623 ± 93	4846 ± 564	1716
27	intermediate	913 ± 38	851 ± 147	78 ± 9	1842 ± 110	396
28	intermediate	104 ± 53	528 ± 105	649 ± 287	1281 ± 427	673
29	Nantes	275 ± 41	311 ± 72	126 ± 13	711 ± 108	447
30	Nantes	25 ± 5	188 ± 24	79 ± 29	292 ± 52	701
31	Nantes	381 ± 70	157 ± 17	51 ± 7	589 ± 76	491
check	commercial	162 ± 13	N/D[b]	82 ± 8	244 ± 20	458

[a]RMSEE = 459 μg g^{-1}. [b]N/D, none detected, <5 μg g^{-1}.

widest variability of total carotenoid concentrations, from 0 to 6277 μg g^{-1} DW, which is comparable to the results in the current work. Other studies have reported carotenoid concentrations in a narrower range of 1.4−2830 μg g^{-1} DW, even for carrots described as "bright orange".[12,38−40]

Raman Analysis of Carrot Powders. Analyses of carrot powders with laser excitation at 830 and 785 nm showed Raman bands at 1520, 1156, and 1006 cm^{-1} (Figure 4). The bands at 1520 and 1156 cm^{-1} have been previously assigned to the in-phase symmetric stretching of −C═C− and −C—C− of the carotenoid polyene chain, respectively.[41] The band at 1006 cm^{-1} is due to the in-plane rocking of the methyl side groups on the carotenoid polyene chain.[41] The intensity of these bands varied with sample carotenoid concentrations, demonstrating the potential of 830 and 785 nm excitation to be used for the quantitation of carotenoids in freeze-dried carrot powders. However, excitations at these laser wavelengths were unable to distinguish the diacetylene key band at 2250 cm^{-1} from background fluorescence of carrot matrix constituents. Even scans of parsnip (*Pastinaca sativa* L.) extract very rich in polyacetylenes (10000 μg g^{-1} by GC-FID) did not distinguish the diacetylene symmetric stretch from background noise. Therefore, analysis of polyacetylenes in carrot powder at these excitation wavelengths was not likely to be viable, at least using the experimental setups described. Spectra of carrot powders at these wavelengths showed a raised baseline that decreased in intensity with an increase in Raman shift from the incident frequency, a strong indication of sample fluorescence (Figure 4).

FT-Raman analysis at 1064 nm proved to be more useful. Fluorescent interference was much reduced, resulting in spectra with lower baselines (Figure 4). These high-quality Raman spectra showed several useful bands not visible using incident beams with shorter excitation wavelengths. The bands have previously been assigned to various symmetric modes of carrot matrix components including carotenoids (1520, 1156, and 1006 cm^{-1}), total carbohydrate (2800−3050 cm^{-1}), lignin (1592 cm^{-1}), starch (450−510 cm^{-1}), pectin (854 cm^{-1}), and polyacetylenes (2250 cm^{-1}) (Figure 4).[28,32,34]

We found that linear Raman dose response curves could be generated for both β-carotene and polyacetylenes by plotting the integral of key Raman bands against the concentration of the relevant phytonutrient dispersed in cellulose powder. Although cellulose is not identical to a carrot matrix, the resulting response curves showed that the carotenoids and polyacetylenes could be quantitated in a plant-like matrix using the intensity of their Raman key bands. For polyacetylenes, an r^2 value of 0.995 was obtained (Figure 2). For β-carotene, an r^2

Table 2. Carotenoid Concentrations across 31 Carrot Lines (Micrograms per Gram DW; Mean of Three Agronomic Replicates ± Standard Deviation) by Visible Spectrophotometry and FT-Raman

			total carotenoids	
line	type	color	GC-FID	FT-Raman[a]
1	Nantes	orange	3758 ± 706	3536
2	intermediate	purple outer/orange inner	3008 ± 345	3099
3	Imperator	orange	3630 ± 437	4239
4	Nantes	orange	4600 ± 1128	3664
5	intermediate	pink	N/D[b]	63
6	Nantes	orange	3500 ± 174	3080
7	intermediate	orange	2894 ± 459	3329
8	Imperator	deep orange	5064 ± 699	4345
9	Nantes/Kuroda	orange	4908 ± 934	3810
10	intermediate	orange	3428 ± 728	3812
11	Old French	white	N/D	0
12	Imperator	orange	3752 ± 850	3802
13	Nantes	orange	3329 ± 619	3482
14	intermediate	yellow	230 ± 37	84
15	Imperator	orange	3842 ± 516	4323
16	Kuroda	orange	3440 ± 342	4040
17	intermediate	purple	326 ± 31	192
18	wild	white	N/D	88
19	wild	white	N/D	207
20	Nantes	pink	N/D	176
21	intermediate	purple	738 ± 92	449
22	Kuroda	yellow	1172 ± 246	1341
23	Flakkee	orange	3521 ± 209	3937
24	wild	white	N/D	97
25	intermediate	yellow	740 ± 82	932
26	wild	white	N/D	0
27	intermediate	deep orange	5586 ± 804	4248
28	intermediate	white	N/D	0
29	Nantes	orange	3679 ± 542	3463
30	Nantes	pink	N/D	128
31	Nantes	orange	4116 ± 698	3733
check	commercial	pale orange	1830 ± 493	2627

[a]RMSEE = 727 μg g^{-1}. [b]N/D, none detected, <100 μg g^{-1} DW.

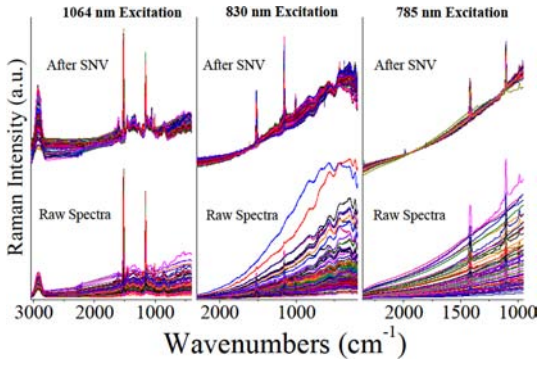

Figure 4. Raman spectra of carrot powders at different wavelengths before (below) and after (above) preprocessing.

value of 0.988 was obtained (Figure 3). Detection limits were 42 and 1400 μg g^{-1} for carotenoids and polyacetylenes, respectively.

We have developed quantitative and semiquantitative chemometric models for the analysis of carotenoids and polyacetylenes based on their key bands using 1064 nm FT-Raman spectroscopy.

Principal Component Analysis (PCA). Following standard normal variate (SNV) preprocessing, PCA was performed on 101 digitized carrot spectra (31 × 3 replicates + 8 check samples). The resulting loading plots indicated that samples in PC space were being described by carotenoid (PC-1) and carbohydrate (PC-2) concentrations in the samples (Figure 5). PC-1 and PC-2 together accounted for 94% of the variability in the Raman spectra. Samples were labeled by their colors to highlight the latent data trend in PC-1, that is, carotenoid content (Figure 5). PC-2 separated samples largely on the basis of the C−H stretching vibration in the region of 2800−3050 cm^{-1} and also showed weaker weighting based on the variance of the spectral regions 450−510 and 854 cm^{-1}, which have previously been assigned to vibrational modes of starch and pectin, respectively (Figure 5).[32,34] The check sample analytical replicates were tightly clustered in the PCA plot, demonstrating the reproducibility of the analyses (Figure 5). Agronomic replicates generally aggregated in PC space, with three distinct groupings of carrot lines (Figure 5). The intensely orange carrots were grouped in positive PC-1 space, whereas the white/pink/purple lines were grouped in negative PC-1 space. Orange carrots spanned a wide range on PC-1, indicative of their varying carotenoid levels. The lines grouped in negative PC-1 space were divided into two clusters by PC-2. Carrot lines in positive PC-2 space had higher carbohydrate contents than those in negative PC-2 space. This indicated that the wild varieties isolated in the negative PC-1 and PC-2 quadrant were lower in carbohydrate than the other albino and carotenoid-deficient lines. Due to the low intensity of the polyacetylene band around 2250 cm^{-1}, carrot samples were not separated on their polyacetylene contents in this indiscriminant PCA. Therefore, a more supervised approach had to be adopted to relate Raman spectra to their polyacetylene concentration.

Quantitative Modeling. The intensities of the three carotenoid bands in the carrot Raman spectra (Figure 3) were individually related to carotenoid concentration by visible spectrophotometry using multiple linear regression (MLR), principal component regression (PCR), and partial least-squares regression (PLS-R). Each regression method was applied to spectral data after either standard normal variate (SNV) or multiplicative scatter correction (MSC) preprocessing had been performed. Performance of the resulting models is summarized in Table 3. The calibration accuracy is described by the r^2 value. The usefulness of the model for accurately predicting carotenoid concentrations is described by the validation r^2 value. Cross-validation was performed in 20 segments each containing 5 randomly selected samples. The most robust models were produced by preprocessing spectra with MSC followed by using MLR to correlate carotenoid data to the Raman intensity at 1520 ± 10 cm^{-1}.

Carotenoid Model Summary (Figure 6)

calibration $r^2 = 0.879$

validation $r^2 = 0.85$

validation RMSEE = 727 μg g^{-1}

The known polyacetylene Raman response at 2250 ± 20 cm^{-1} was related to total polyacetylenes, measured by GC-FID,

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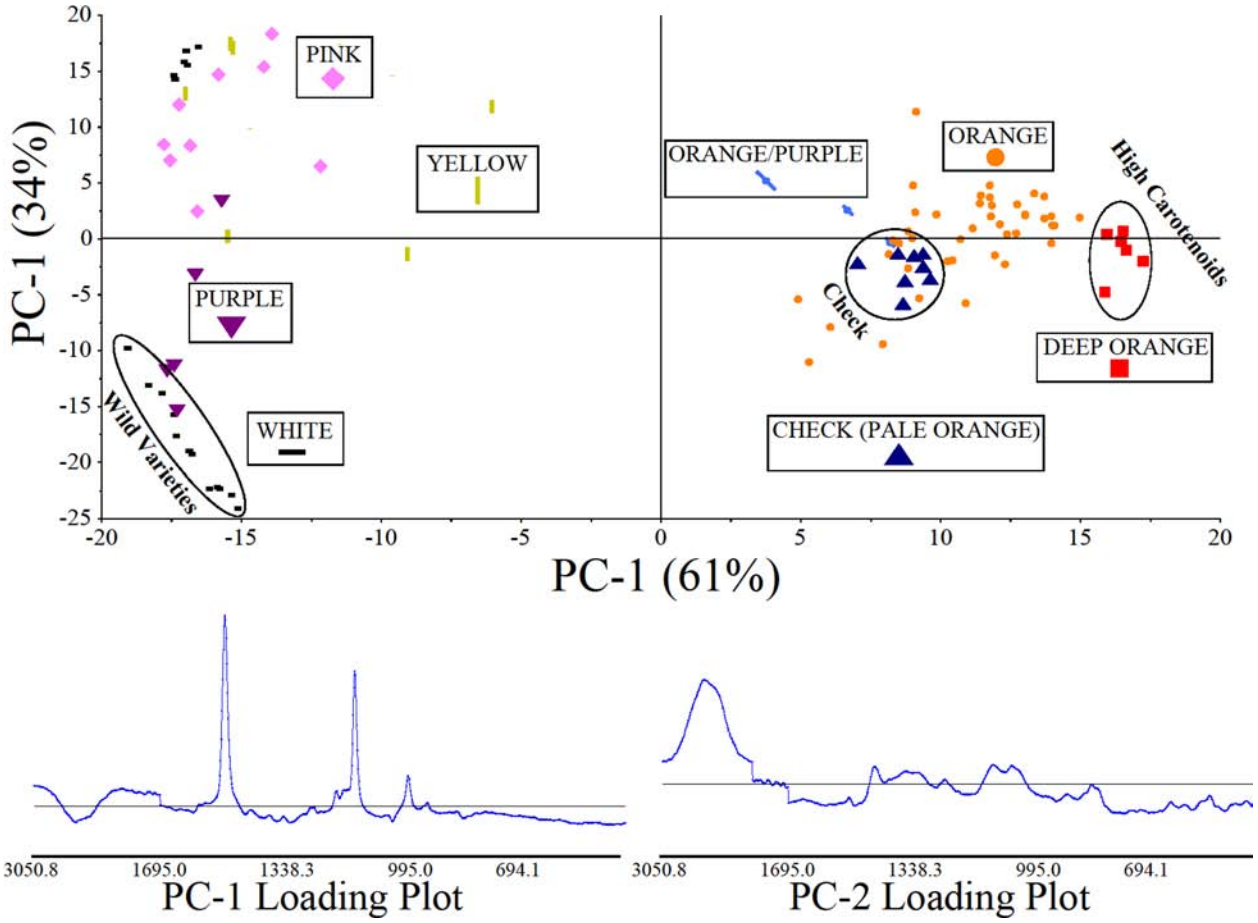

Figure 5. Scores plot showing the distribution of carrot samples in PC space with inset spectral loadings plots describing 1064 nm FT-Raman spectral variance.

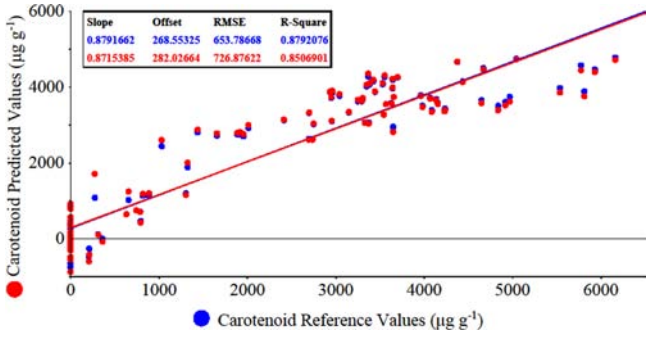

Figure 6. Multiple linear regression plot of carotenoid peak at 1520 cm^{-1} versus carotenoids by UV–vis.

with the same statistical techniques of MLR, PCR, and PLS-R. Each technique was applied to spectral data that were first preprocessed using MSC and SNV. Model performance is summarized in Table 3. Cross-validation was performed as outlined for carotenoids. Preprocessing by MSC followed by MLR correlation resulted in the most useful model.

It can be seen that polyacetylene prediction models perform better when MSC is used instead of SNV. Due to the diversity of carrots in the study and the extreme Raman activity of carotenoids, spectral diversity is extreme. Because SNV normalizes spectra using the standard deviation of spectral points from a *single* spectrum, the technique is prone to the introduction of nonlinearities.[42] MSC, on the other hand, normalizes spectra using the average spectrum of the population, rendering the diversity of spectral features moot. As such, this study provides an excellent evaluation of the limitations of SNV for the analysis of a minor spectral feature in the presence of a prominent, varying spectral feature.

Polyacetylene Model Summary (Figure 7)

calibration = 0.568

validation r^2 = 0.324

validation RMSEE = 549 μg g^{-1}

The root mean square error of estimation (RMSEE) gives a value figure in the original units ($\mu g\ g^{-1}$) describing the modeling error. In Figures 6 and 7 the calibration r^2 values are represented by the blue lines and the validation r^2 values by the red lines.

The r^2 value for the carotenoid model (0.879) indicated a good predictive model that correlated well with the results by spectrophotometry. The r^2 value of the polyacetylene model (0.568) showed that, although there was some relationship between the spectral range from 2250 ± 10 cm^{-1} and polyacetylene concentration, the model had weak or no quantitative predictive power. This was not surprising as the polyacetylene concentrations of most of the samples fell below the LOD for polyacetylenes (as calculated in cellulose powder). The model could, however, be used to distinguish carrots containing high polyacetylene concentrations from other lines

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Table 3. Quantitative Model Performance for Various Preprocessing and Regression Techniques

preprocessing	regression	ν range (cm^{-1})	calibration r^2 (no. of factors/PCs)	validation (r^2)
SNV	MLR	1006 ± 10[a]	0.84	0.81
		1157 ± 10[a]	0.86	0.83
		1520 ± 10[a]	0.88	0.85
		2250 ± 10[b]	0.44	0.42
	PCR	1006 ± 10[a]	0.84 (3)	0.83
		1157 ± 10[a]	0.83 (1)	0.83
		1520 ± 10[a]	0.82 (1)	0.82
		2250 ± 10[b]	0.29 (2)	0.25
	PLS-R	1006 ± 10[a]	0.83 (2)	0.83
		1157 ± 10[a]	0.83 (1)	0.82
		1520 ± 10[a]	0.87 (4)	0.86
		2250 ± 10[b]	0.29 (2)	0.24
MSC	MLR	1006 ± 10[a]	0.85	0.84
		1157 ± 10[a]	0.86	0.83
		1520 ± 10[a]	**0.88**	**0.85**
		2250 ± 10[b]	**0.57**	**0.32**
	PCR	1006 ± 10[a]	0.84 (3)	0.83
		1157 ± 10[a]	0.85 (1)	0.84
		1520 ± 10[a]	0.83 (1)	0.83
		2250 ± 10[b]	0.44 (2)	0.41
	PLS-R	1006 ± 10[a]	0.84 (4)	0.83
		1157 ± 10[a]	0.85 (1)	0.84
		1520 ± 10[a]	0.83 (1)	0.83
		2250 ± 10[b]	0.45 (2)	0.42

[a]Carotenoid band. [b]Polyacetylene band.

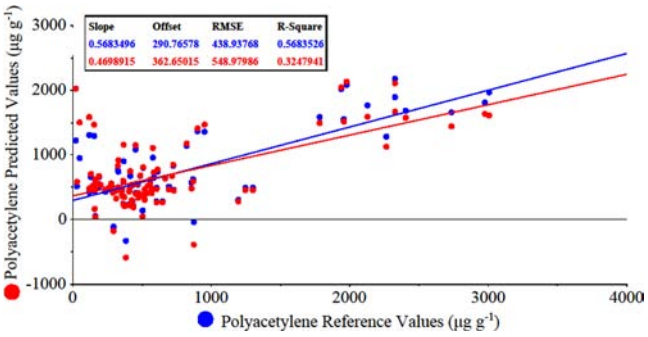

Figure 7. Multiple linear regression plot of polyacetylene peak at 2250 cm^{-1} versus total polyacetylenes by GC-FID.

to some extent and as such may have value to breeders selecting for this trait.

We have shown that NIR FT-Raman can be used to determine carotenoid concentrations in lyophilized carrots. This technique requires considerably less time than traditional analysis methods, which require a solvent extraction prior to analysis. The model generated here encompasses white to bright orange carrots and as such should be relevant to the majority of carrot varieties in cultivation. Quantitation of polyacetylenes in carrots by NIR FT-Raman spectroscopy is possible but only to a limited extent, because the concentrations of polyacetylenes in the majority of carrots are below the limit of detection. It can, however, differentiate carrots with high polyacetylene concentrations (>3000 $\mu g\ g^{-1}$) from those with normal/low levels. As such, the technique may be useful for breeders identifying carrots with high polyacetylene concentrations, which was the original aim of this research. This study encompasses an extremely wide range of carrot genetic variability and provides a useful guide to the variability of carotenoid and polyacetylene concentrations in carrots. It is demonstrated that chemometrics in conjunction with Raman spectroscopy has the potential to be used for the semi-quantitative analysis of polyacetylenes and quantitative analysis of carotenoids in carrots. Furthermore, it is shown that FOH, **1**, is the majority polyacetylene in most carrot varieties, whereas FDOH3Ac, **3**, is usually the minority component.

We have shown that Raman spectroscopy in conjunction with chemometrics can simultaneously analyze two very different classes of natural products. Whereas Raman spectroscopy does not provide concentrations of individual compounds, unlike LC or GC, no solvent extraction or cleanup is needed. The methods and models described here can be used as a bioactivity compass, directing breeders to carrot lines with desirable traits in a workable time frame. This makes Raman spectroscopy ideal for the initial screening of numerous samples.

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