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Comparison of Polyacetylene Content in Organically and Conventionally Grown Carrots Using a Fast Ultrasonic Liquid Extraction Method

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A rapid and sensitive analytical method for quantification of polyacetylenes in carrot roots was developed. The traditional extraction method (stirring) was compared to a new ultrasonic liquid processor (ULP)-based methodology using high-performance liquid chromatography−ultraviolet (HPLC−UV) and mass spectrometry (MS) for identification and quantification of three polyacetylenes. ULP was superior because a significant reduction in extraction time and improved extraction efficiencies were obtained. After optimization, the ULP method showed good selectivity, precision [relative standard deviations (RSDs) of 2.3−3.6%], and recovery (93% of falcarindiol) of the polyacetylenes. The applicability of the method was documented by comparative analyses of carrots grown organically or conventionally in a 2 year field trial study. The average concentrations of falcarindiol, falcarindiol-3-acetate, and falcarinol in year 1 were 222, 30, and 94 μg of falcarindiol equiv/g of dry weight, respectively, and 3−15% lower in year 2. The concentrations were not significantly influenced by the growth system, but a significant year−year variation was observed for falcarindiol-3-acetate.

KEYWORDS: Carrots (*Daucus carota*); conventional and organic growth systems; HPLC−UV and MS; method development and validation; polyacetylenes; ultrasonic liquid processor (ULP)

INTRODUCTION

Epidemiological studies have shown that a high intake of fruits and vegetables improves the protective effects against cancer (*1*−*3*) and cardiovascular diseases (*4*). Carotenoids are believed to play an important role in the health-promoting properties of, e.g., carrots (*5*), although the protective effect of carotenoids on certain types of cancer can be attenuated or reversed by smoking (*6*, *7*). In addition, it has been suggested that other less abundant bioactive compounds, such as the aliphatic polyacetylenes, might contribute significantly to the potential positive effects of carrot consumption (*8*).

Polyacetylenes are toxic to certain fungi (*9*) and other pathogens (*10*), have anti-inflammatory effects in macrophage and endothelial cells (*11*), and can cause strong allergenic reactions (*12*). They have protective effects against various cancer cells (*13*, *14*), including colorectal cancer in rats (*15*), and the response appears to be dose-dependent (*16*). It has also been shown that falcarinol is readily bioavailable to humans (*17*).

More than 1400 different polyacetylenes are found in plants, and they are believed to be synthesized from unsaturated fatty acids. The aliphatic C_{17}-polyacetylenes are widely distributed in, e.g., carrots, celery, and parsley (*8*), all belonging to the Apiaceae family. Carrots are the major human dietary source of polyacetylenes (*15*), and the chemical structures of the main compounds found in carrots are shown in **Figure 1**. The content in carrots depends upon factors such as cultivar (*5*, *18*, *19*), physiological age, geographical origin (*5*), and climatic conditions, e.g., plant-available water content of soils (*20*), as well as storage and processing procedures (*21*). The amount of plant-available nitrogen might also be of importance, as seen for many other secondary metabolites, such as carotenoids (*22*) and flavonoids (*23*). The spatial distribution of individual polyacetylenes in a carrot root differs. Falcarinol appears to be uniformly distributed, whereas falcarindiol and falcarindiol-3-acetate are primarily found in the peel (*9*, *19*, *24*).

Polyacetylenes in carrots have previously been extracted from fresh or dried plant material with ethyl acetate (EtOAc) as the most frequently used extraction solvent (*9*, *18*, *21*, *24*, *25*). Extraction of lyophilized plant material has been shown to yield similar results in comparison to fresh plant material (*18*). The traditional liquid extraction methods include stirring (*24*) and ultrasonication (*14*), but pressurized liquid extraction has also recently been used (*18*). The risk of degradation is increased with

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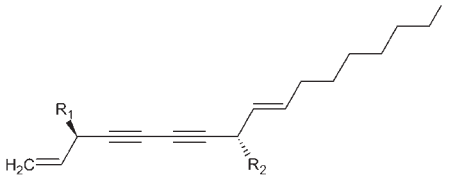

	Abbreviation	R_1	R_2
Falcarinol [a]	FaOH	OH	H
Falcarindiol [b]	FaDOH	OH	OH
Falcarindiol-3-acetate [c]	FaDOAc	$OCOCH_3$	OH

[a] 1,9-heptadecadiene-4,6-diyne-3-ol
[b] 1,9-heptadecadiene-4,6-diyne-3,8-diol
[c] 1,9-heptadecadiene-4,6-diyne-3,8-diol, 3-acetate

Figure 1. Chemical structures of the main polyacetylenes in carrots. The systematic names are listed in the footnotes.

long extraction times because of the instability of polyacetylenes, especially falcarindiol, which is sensitive to oxidation and/or enzymatic degradation (*26*) as well as intolerant to heat and light exposure (*21*). Ultrasonic liquid processing (ULP) is a promising technique, which has not previously been used for extraction of polyacetylenes from carrots. The advantage of ULP is that shorter extraction times are expected because of a more intense cavitational action and greater disruption of the sample.

The chemical analysis of polyacetylenes is usually performed on a high-performance liquid chromatography (HPLC) system equipped with a reversed-phase column using gradient elution with methanol (MeOH) or acetonitrile (MeCN) and water, but gas chromatography has also been used. Ultraviolet (UV) detection is used for quantification because polyacetylenes yield characteristic UV spectra because of their conjugated triple bonds, but their extinction coefficients are generally low at UV maxima because of the low number of conjugated unsaturated bonds. Hence, the quantification is often performed at 205 nm, where the sensitivity is 10 times higher than at their characteristic UV maxima (*8*). Quantification by mass spectrometry (MS) has also been applied (*18*) and is especially relevant when measuring small concentration levels in, e.g., blood plasma, because of its high sensitivity (*8*).

The objectives of the current study were to identify polyacetylenes in carrot roots by application of HPLC−MS and to develop a faster, more sensitive, and robust analytical method based on ULP extraction and HPLC−UV quantification. The developed ULP method was optimized and compared to the traditional extraction by the stirring protocol. The applicability of the method was demonstrated by comparative analysis of organically and conventionally grown carrots to study the effect of the growth system on the concentration of polyacetylenes in carrot roots. Organic carrots are produced and consumed in large quantities in Denmark, and possible health benefits of organic foods in general are of major interest to, e.g., consumers and producers.

MATERIALS AND METHODS

Reagents and Chemicals. Methanol (MeOH), ethyl acetate (EtOAc), and acetonitrile (MeCN) were HPLC-grade (Rathburn Chemicals Ltd., Walkerburn, Scotland). Dimethyl sulfoxide (DMSO) was >99%, and formic acid was 98−100% (Merck, Darmstadt, Germany). Milli-Q water, 18 MΩ (Millipore, Bedford, MA), was used for sample preparation and eluents.

Falcarindiol, yellowish oil, was 97% purity (Atomax Chemicals Co., Ltd., Shenzhen, China), and a standard stock solution (1 mg/mL) was prepared by dissolving the compound in MeOH. Working solutions of falcarindiol (20 μg/mL) were prepared by diluting the stock solutions with MeOH. Stock solutions of falcarindiol were stored (<1 year) at −80 °C, while working solutions were prepared shortly before analyses and kept at −20 °C until analyses.

Samples for Method Development, Optimization, and Validation. A conventionally grown carrot sample (*Daucus carota* cv. 'Bolero', 50−250 g of fresh weight, Lammefjorden, Faarevejle, Denmark) was purchased at a local supermarket and used for comparison of extraction methods, ULP method optimization, and validation. An in-house carrot sample was used as reference material for method validation together with randomly selected carrot samples from the field trial experiment (described below). The samples were washed in Milli-Q water, peeled, cut into 0.5 cm thick slices, and freeze-dried at 0.1 kPa for 2 days (Beta 1-8, Christ, Osterode am Harz, Germany). Afterward, the samples were crushed, homogenized, and stored at −20 °C in an inert nitrogen atmosphere until analysis. In general, the samples were protected from light and oxygen during the entire sample preparation by wrapping in aluminum foil and storing in a nitrogen atmosphere.

Samples from the Field Trial Experiment. The applicability of the method was documented by comparative analyses of organically and conventionally grown carrot roots from a 2 year field trial study undertaken in 2007 (year 1) and 2008 (year 2). The carrots (*D. carota* cv. 'Bolero') were grown in the VegQure rotation experiment (*27*) located at the Aarslev field trial station, Funen, Denmark (10°27′E, 55°18′N). A sandy loam soil (15% clay, 27% silt, and 55% sand) with pH 6.2 (measured in 0.01 M $CaCl_2$) was used. The soil had been adapted to organic production for a decade and plant-available, and plant-available P, K, and Mg were 2.6, 12.4, and 4.3 mg/100 g of soil, respectively (average value for plough layer soil samples taken in March in both growth years), measured in soil samples using standard procedures (*28*).

The carrots were grown in three different agricultural systems: one conventional (C) and two organic (OA and OB) growth systems with three replicates of each in the field, resulting in 9 plots per year and 18 plots in total (each of 120 m^2). The replicates from each system were located in three separate blocks geographically close to each other. Each block contained all three agricultural systems, which were all stock-less cash crop production systems with an identical sequence of main crops (8 year rotation). In the conventional system (C), pesticides and inorganic fertilizer were used (120, 18, and 58 kg of N, P, and K/hectare, respectively). The OA system relied on fertilization with animal pig manure (54, 4, and 20 kg of N, P, and K/hectare, respectively), while the nutrient supply was based on the use of cover crops as green manures (mainly legumes) in the OB system. Cover crops were grown in the autumn after the main crops and incorporated into the soil in the spring before carrots were grown. The organic systems were managed in full compliance with the Danish guidelines for organic farming administered by the Danish Plant Directorate (*29*).

During the growth period (from the end of May to the beginning of October), a climatic year−year variation was observed. The average temperatures in years 1 and 2 were 15.4 and 15.1 °C, respectively, while precipitation was ∼60% higher in year 1. However, this difference was balanced by intensive irrigation in year 2. The carrots were harvested at maturity at the same day from all growth systems. A 15 kg sample was collected from each plot with 50−250 g of fresh weight of individual roots (marketable quality) as inclusion criteria in both growth years. Sampling representativeness was tested by double sampling of random plots and yielded an average relative standard deviation (RSD) of 8.9% for falcarindiol, falcarindiol-3-acetate, and falcarinol (n = 6 for each compound).

Only the edible parts of the carrots were analyzed, and the samples were prepared for analysis as described above. However, samples from the field trial experiment were freeze-dried at 0.08 kPa for 1−2 days at a commercial freeze-drying company (Danish Freeze-Dry A/S, Kirke Hyllinge, Denmark).

Extraction Methods. EtOAc was used as the extraction solvent throughout the method comparison and in the final optimized method.

Extraction Method with Stirring. A total of 0.5 g of freeze-dried material was extracted with 30 mL of EtOAc overnight under continuous stirring at room temperature. Before stirring, the sample was exposed to an ultrasound for 10 min, 120 W, Brasonic 5200 (Soest, The Netherlands). The extract was filtered, and the residue was further extracted with 30 mL of EtOAc for 3 h. The combined extracts were evaporated to dryness *in vacuo* at 30 °C on a rotary evaporator. The extraction procedure was based on the principles suggested by Christensen and Kreutzmann (*24*).

Extraction with ULP. A total of 0.5 g of freeze-dried material (1 g in the optimized ULP method) was transferred to a cylindric plastic tube, and 30 mL of EtOAc was added. The microprobe was immersed into the tube, and the extraction was performed at room temperature for 60 s using an ULP, Microson XL 2000 (Misonix, Newtown, CT), operated at 10 W. The sample was centrifuged for 5 min, 6000 rpm, in a Varifuge RF (Heraeus), and 20 mL of the supernatant was evaporated to dryness *in vacuo* at 30 °C on a rotary evaporator.

Residues from both extraction methods were redissolved in MeOH (5 mL) and filtered through a 0.45 and a 0.20 μm filter before chemical analysis.

Quantification of Polyacetylenes by HPLC−UV. A Waters 2695 Alliance Separations Module in combination with a Waters 2996 photodiode array detector (PDA) and a Waters 2487 dual λ absorbance detector (Waters, Milford, MA) was used for the chromatographic analysis of polyacetylenes. Empower 2 was used for instrument control and data acquisition. The chromatographic separation was carried out at a flow rate of 1.0 mL/min at 40 °C with an injection volume of 15 μL (temperature of the autosampler of 5 °C). The column was a Prodigy RP-C18 column, 4.6 × 250 mm, 5 μm (Phenomenex, Torrance, CA). The A and B eluents were Milli-Q water and MeCN, respectively. The gradient program was as follows: 70% B for 5 min, a linear gradient to 86% B for 13 min, a linear gradient to 95% B for 2 min, isocratic elution for 8 min, followed by a 2 min ramp back to 70% B and re-equilibration for 3 min, giving a total run time of 33 min. The PDA collected data from 190 to 400 nm and the dual λ absorbance detector were used for quantification of the polyacetylenes (205 nm), which were quantified relative to falcarindiol, because this was the only commercially available standard. The structures of the three polyacetylenes are quite alike and were anticipated to have rather similar UV spectra and absorptivities, justifying the use of equivalency for quantifying and comparing crops from different cultivation systems. The purity of the standard was reported to >97% and confirmed by HPLC analysis (UV detection at 205 nm).

Identification of Polyacetylenes by HPLC−UV and MS. Structure elucidation of polyacetylenes in carrots was based on accurate mass measurements, isotopic pattern fit of the measurement compared to the theoretical (i-FIT) values, Elemental Composition 4.0 software (Waters, Milford, MA), and fragmentation patterns. An ultra-performance liquid chromatograph (UPLC) interfaced to a time-of-flight tandem mass spectrometer (TOF−MS) was used for exact mass determinations. Afterward, the identities were confirmed with the available standard by a comparison of retention times and UV and MS data. UV spectra of the common polyacetylenes from carrots show three absorption maxima at about 230, 245, and 260 nm (*8*, *20*, *24*).

UPLC−TOF. The same mobile phases as those used for the HPLC−UV analyses were applied for the UPLC analyses, Acquity UPLC (Waters, Milford, MA), except the addition of 0.1% formic acid to the A eluent to enhance ionization. This change in solvent had no significant effects on the retention times. The separation of analytes was performed at 40 °C on an Acquity HSS C18 column, 2.1 × 100 mm, 1.8 μm (Waters, Milford, MA). The HPLC gradient was scaled according to the column dimensions, and gradient flow rates were adjusted to the UPLC mode. The gradient program was as follows (0.25 mL/min): 70% B for 1.7 min, a linear gradient to 86% B for 4.3 min, a linear gradient to 95% B for 0.7 min, isocratic elution for 2.9 min, followed by a 1.2 min ramp back to 70% B and re-equilibration for 3.6 min, giving a total run time of 14.4 min. A sample volume of 3 μL was injected using the partial loop with needle overfill mode. The TOF−MS, LCT Premier/XE mass spectrometer (Waters, Milford, MA) was operated in electrospray ionization positive-ion mode (ESI^+). The operating conditions were ion source temperature, 120 °C; desolvation gas (N_2), 350 °C, 500 L/h; cone gas, 25 L/h; capillary voltage, 2 kV; and scan time, 1.0 scan/s. MassLynx software, version 4.1, was used for instrument control and data acquisition, and leucine enkephaline (556.2771 g/mol) was used as the lock-spray mass (reference standard). External mass calibration (m/z 100−1000) was carried out before the analyses using a sodium formate solution containing 5 mM sodium hydroxide and 0.5% formic acid in 2-propanol/water (90:10, v/v).

Method Optimization and Validation. *Optimization of Extraction with ULP*. The following ULP parameters were optimized: sample weight (0.5 and 1.0 g of freeze-dried material), extraction time (15, 60, and 120 s), and number of extractions (1, 2, and 3 times). They were optimized with regard to the extraction efficiency (the quantity of extractable polyacetylenes per unit weight), precision (determined as the RSD from a number of replicate analyses measured under repeatable conditions), and sensitivity (the capability of the method to discriminate small concentration differences of the polyacetylenes).

Optimization of the Chromatographic Method. The HPLC method used by Christensen and Kreutzmann (*24*) was optimized by gradient adjustments to reduce the total run time. A pilot study was also conducted to test the applicability of a faster chromatographic technique, UPLC (Waters, Milford, MA), with a PDA detector for quantification at 205 nm. The columns tested were Acquity UPLC BEH C18, 2.1 × 50 mm, 1.8 μm, and Acquity UPLC HSS C18, 2.1 × 50, 100, and 150 mm, 1.8 μm (Waters), which were retained at 40 °C. The same mobile phases as those used for the HPLC−UV analyses were used, and the gradient was scaled and optimized according to the column dimensions and flow rates applied (0.2−0.8 mL/min). The best achievable separation with regard to resolution and speed was obtained on the HSS C18 100 mm column at 0.2 mL/min with the following gradient: 70% B for 2 min, a linear gradient to 86% B for 8 min, a linear gradient to 95% B for 2.5 min, isocratic elution for 1.5 min, followed by a 0.5 min ramp back to 70% B and re-equilibration for 2.5 min, giving a total run time of 17 min.

Method Validation of the ULP Method. The selectivity was studied by extraction of blank samples (i.e., extractions of reagents only, without the addition of carrot material) because carrot samples without polyacetylenes were not available. The precision was determined by triplicate analyses of the same sample in three series (i.e., repeatability of measurements of the same sample using the same method on various days). The sensitivity and linear range of measurement were determined for the standard falcarindiol as well as falcarindiol, falcarindiol-3-acetate, and falcarinol in a sample by varying the injection, which provided comparable results to serial dilution of standards. The limit of detection (LOD) and quantification (LOQ) was determined as the intercept of the standard curve plus 3 and 10 times, respectively, the standard deviation of the intercept. The accuracy of the method was determined by recovery experiments because no certified reference samples or samples with or without a low concentration of the polyacetylenes were available. The recovery was determined by extraction of triplicates in three series with and without the standard addition of falcarindiol, which was the only commercially available standard. We did not use an internal standard because it was not possible to find a suitable reference standard, which had similar chemical and physical properties, it did not coelute with the compounds of interest, and it was not present in the matrix.

Quality assurance was performed by including an in-house carrot sample as the reference material (average RSD of 2.8%; $n = 3$) and duplicate measurements (including weighing and extraction) of randomly selected samples in each series of analyses, yielding an average RSD of 4.6% for falcarindiol, falcarindiol-3-acetate, and falcarinol ($n = 9$ for each compound).

Elemental Nitrogen Analysis. Nitrogen was measured using isotope ratio mass spectrometry (IR−MS, Europa Scientific, Crewe, U.K.). Approximately 4 mg of pulverized material was weighed in tin capsules and introduced to the MS via a combustion interface. Quality assurance was performed using certified reference material, frequent quality control samples, as well as duplicate measurements of all samples.

Statistical Analysis. The responses y_{ysb} were modeled as $y_{ysb} = \mu + \alpha_b + \beta_y + \delta_s + \varepsilon_{ys} + \varepsilon_{yb} + \varepsilon_{ysb}$, where μ is the generalized intercept, α_b, with b = 1, 2, and 3, is the effect of the blocks, β_y, with y = years 1 and 2, is the effect of the year, and δ_s, with s = C, OA, and OB, is the effect of the growth system. Errors (ε) are considered independently and normally distributed and represent corresponding variance components of interaction. The pair-wise comparisons and their confidence intervals between the systems were adjusted to obtain a family-wise error rate of 5%. The model was fitted using the proc-mixed procedure in the software packages SAS/STAT, version 9.2 (SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

Identification of Polyacetylenes in Carrots. A HPLC chromatogram and the corresponding UV spectra confirmed the presence of three aliphatic C_{17}-polyacetylenes (falcarindiol, falcarindiol-3-acetate, and falcarinol) in carrots (**Figure 2**), in agreement with previous findings (*8*). Three absorption maxima were observed in

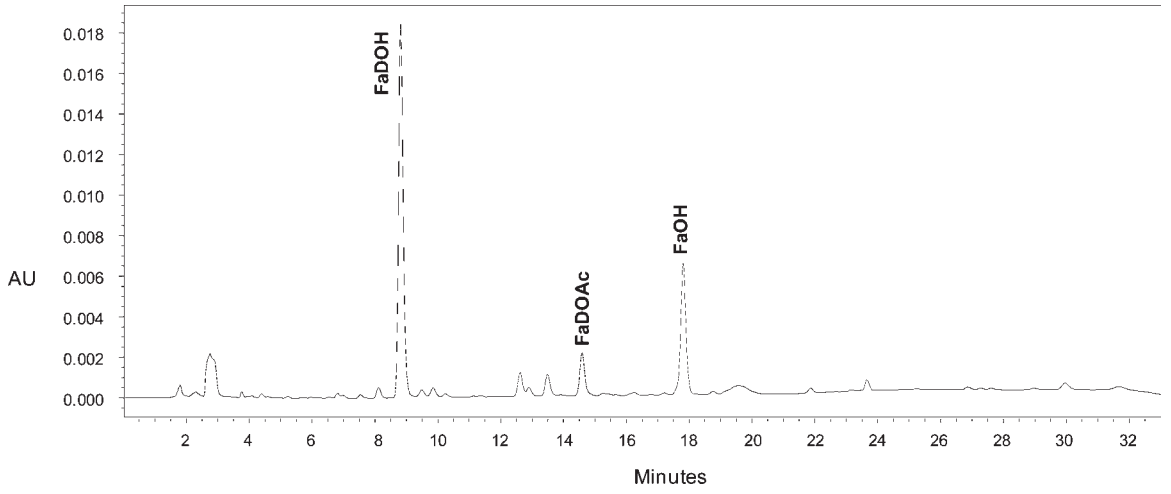

Figure 2. Chromatogram of a carrot extract (HPLC−UV, 205 nm).

Table 1. Harvest Yield Expressed as Tons of Fresh Weight/Hectare (Ton of fw/ha), Carrot Root Size (g of fw/Peeled Carrot), Nitrogen Content (% in Dry Matter), and Dry Matter Content (%) in Three Different Growth Systems (C, Conventional; OA, Organic Using Animal Manure; and OB, Organic Using Cover Crops) and in Two Harvest Years (1 and 2)[a]

	harvest yield (ton of fw/ha)		carrot size (g of fw/carrot)		N (% in dry matter)		dry matter (%)	
	year 1	year 2	year 1	year 2	year 1	year 2	year 1	year 2
C	94.7 ± 3.2	106 ± 4.4	98.1 ± 4.4	82.2 ± 5.0	0.93 ± 0.08	0.96 ± 0.12	11.8 ± 0.2	11.3 ± 0.2
OA	84.8 ± 5.6	99.1 ± 4.8	91.9 ± 5.6	85.5 ± 15	0.71 ± 0.05	0.71 ± 0.10	11.5 ± 0.1	11.5 ± 0.2
OB	89.5 ± 4.9	92.4 ± 4.1	110 ± 12.3	94.6 ± 13	0.80 ± 0.04	0.80 ± 0.03	11.8 ± 0.1	11.5 ± 0.2
year effect	$p = 0.13$		$p = 0.28$		$p = 0.84$		$p = 0.31$	
system effect	$p = 0.26$		$p = 0.015$ C b, OA b, OB a		$p = 0.0005$ C a, OA b, OB b		$p = 0.76$	

[a] The averages ± standard deviations are shown ($n = 3$). The two lower rows represent the results from the statistics across growth systems (year effect) or years (system effect). Growth systems followed by different letters are significantly different ($p < 0.05$).

the UV spectra of the identified polyacetylenes at 230−234, 243−246, and 257−260 nm, in agreement with previous obtained spectra of the same compounds (*20*, *24*).

The MS analysis showed that the identified polyacetylenes had low abundance of the protonated molecular ions $[M + H]^+$ (**Table 1**). The relative abundances of the $[FaDOH + H]^+$ ion and the $[FaDOAc + H]^+$ ion were 2 and 9%, respectively. The $[FaOH + H]^+$ ion was not observed in ESI^+ mode, which is the most appropriate mode compared to ESI^-, where polyacetylenes are non-detectable (*18*). Fragments because of the loss of one water molecule from the protonated molecular ion, $[M - H_2O + H]^+$, of falcarindiol and falcarindiol-3-acetate had abundances of 75−100% and more modest 21% for falcarinol fragments. In addition, the relative abundance of the falcarindiol $[M - H_2O - OH]^+$ ion was 55%, and the relative abundance was 69% for the $[M - AcO]^+$ ion of falcarindiol-3-acetate. Adducts of MeCN, $[M - H_2O + CH_3CN + H]^+$, showed relative abundances of 100% for falcarindiol and falcarinol, in accordance with a previous study of polyacetylenes using atmospheric pressure chemical ionization (APCI) in positive mode and a MeOH eluent (*30*). The latter adduct has also previously been observed as the most abundant ion of falcarinol in ESI^+, and the formation of MeCN or MeOH adducts seems to be typical for aliphatic C_{17}-polyacetylenes in positive APCI or ESI ion mode (*18*). Because of the low abundance of the protonated molecular ions, the exact masses of the individual polyacetylenes were determined on the basis of the fragment $[M - H_2O + H]^+$ and they were within an acceptable range (< 5 ppm) from the theoretical values.

Comparison of Extraction Methods. The extraction efficiencies with ULP were 8−15% higher than extraction with stirring, while the sensitivities of these two methods were comparable but can probably be improved by increasing the sample weight. The precisions were similar and in an acceptable range (RSDs of 1−5%) for this type of analysis. The slightly higher extraction efficiencies with ULP could be because it is a very fast method (60 s extraction) in comparison to the traditional methodology with stirring and long extraction times (overnight extraction and extra 3 h). Thereby, the risk of analyte degradation is reduced because of the sensitivity of polyacetylenes toward oxidation (*26*) as well as heat and light (*21*).

Ultrasonication has previously been used for extraction of polyacetylenes (*14*). However, it was not tested in this study because it resembles the same basic principle as used in ULP, but ULP is a much faster extraction method and also expected to cause stronger tissue disruption than ultrasonication. Pressurized liquid extraction has also recently been used (*18*) and could be advantageous for high-throughput applications, where larger sample sets are handled. However, considering the size of the sample set in this study, we considered ULP to be a better choice for extraction of polyacetylenes because of its advantages with regard to efficiency and speed.

Method Optimization and Validation. Extraction times of 15, 60, and 120 s were tested using the ULP; however, no improvements for falcarindiol, falcarindiol-3-acetate, and falcarinol were observed at increasing extraction times, and the precisions were similar (RSDs of 0.7−4.0%). An increase in sample weight from 0.5 to 1.0 g increased the method sensitivity without affecting the extraction efficiency and precision (data not shown). It was also found that in one extraction cycle more than 95% of the extractable polyacetylenes were detected with acceptable precision (RSDs of

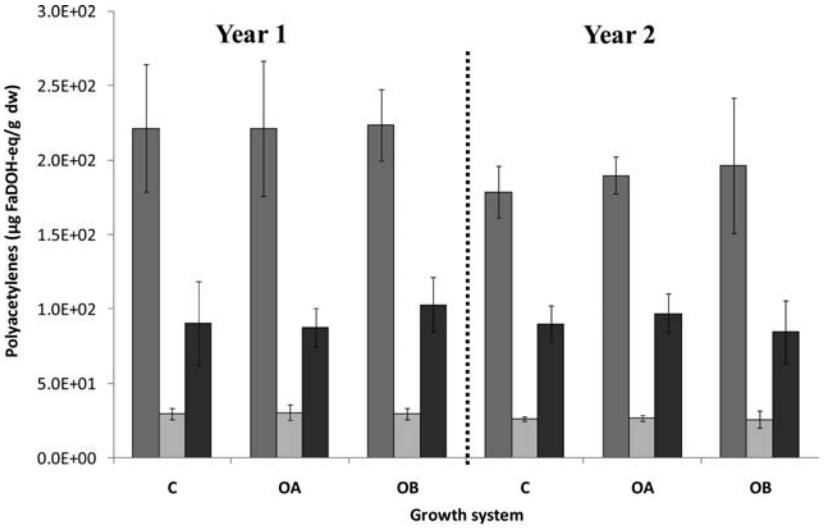

Figure 3. Average concentrations (μg of falcarindiol equiv/g of dry weight) of falcarindiol (medium gray), falcarindiol-3-acetate (light gray), and falcarinol (dark gray) in three different growth systems (C, conventional; OA, organic using animal manure; and OB, organic using cover crops) and in two harvest years (1 and 2). The error bars describe the standard deviation of replicates from the field (n = 3).

1.7−6.0%), while less than 4 and 1% of the polyacetylenes were detected in the second and third extracts, respectively (data not shown). In contrast, two and three extraction cycles were considered necessary in previous studies using extraction with stirring (*14*) and ultrasonication (*24*). Therefore, the optimum extraction conditions were 1.0 g of sample and one extraction cycle of 60 s, whereby more than 95% of the extractable polyacetylenes were obtained with acceptable precision and sensitivity.

A good chromatographic separation of the three polyacetylenes was achieved within 33 min (**Figure 2**), yielding a satisfactory resolution between the compounds of interest and interfering peaks. The marked decrease in chromatographic run time represents a significant analytical improvement in comparison to earlier studies, where run times from 45 to 95 min have been applied (*14*, *18*, *24*).

A pilot study of UPLC resulted in a further reduction in run time by a factor of 2 from 33 to 15 min (data not shown). However, the selectivity of the HPLC and UPLC columns were different, and interfering peaks disturbed the separation in the UPLC chromatogram produced, yielding poor resolution of falcarindiol and falcarindiol-3-acetate. Thus, it was decided to continue with the HPLC separation, because a compatible UPLC column with a similar stationary phase as the one used in the HPLC analysis was not commercially available.

The linear range of measurement for falcarindiol was 0.3−58 μg/mL (equal to 4.5−870 μg/g of dry weight; $R^2 > 0.999$). Because of the structural similarity, it was assumed to be in the same range for falcarindiol-3-acetate and falcarinol when tested by varying the injection volume of a sample, but exact concentration levels could not be obtained because of the lack of available standards. The sensitivity for falcarindiol was 8.6×10^4 AU/(μg/mL), and the selectivity was satisfactory because interfering compounds (coeluting compounds or adduct ions) were neither immediately detected in blank samples nor in the alternative UPLC−MS analysis. The sensitivity and selectivity were fully acceptable for the current study, and a more sensitive and selective detection principle, e.g., MS, was not necessary, as reported in previous studies (*18*).

LODs and LOQs ranged from 3.6 to 7.2 μg of falcarindiol equiv/g of dry weight and from 7.8 to 49 μg of falcarindiol equiv/g of dry weight, respectively. Comparable LODs and LOQs were obtained when adjusting for a possible matrix effect by diluting the precision samples to a concentration close to the expected LOQ and determination of LOD and LOQ as 3 and 10 times the standard deviation. The LOQs were within the linear range of measurement, and the precisions were acceptable for our purpose (RSD of 2.3−3.6%).

Recovery experiments with falcarindiol were used to estimate the trueness of the method because no certified reference samples or samples with or without a low concentration of the polyacetylenes were available and falcarindiol was the only commercially available standard. The recovery of falcarindiol was 93%, which we consider satisfactory. Furthermore, as previously stated, the ULP method yielded satisfactory extraction efficiencies compared to the stirring-based method and more than 95% of the extracted polyacetylenes was detected within one extraction cycle.

Polyacetylenes in Organically and Conventionally Grown Carrots. The developed analytical method was used to compare the content of polyacetylenes in organically and conventionally grown carrot roots. The average concentrations of polyacetylenes across growth systems (±standard deviation, n = 9) in year 1 were 222 ± 33, 30 ± 3.7, and 94 ± 19 μg of falcarindiol equiv/g of dry weight of falcarindiol, falcarindiol-3-acetate, and falcarinol, respectively (**Figure 3**), which corresponds to 26 ± 4.0, 3.6 ± 0.4, and 11 ± 2.3 μg of falcarindiol equiv/g of fresh weight. The concentration levels were 15, 12, and 3% lower in year 2, respectively, and generally in agreement with previously reported values (*5*, *14*, *18*, *19*, *25*). However, the concentration of falcarindiol in peeled carrots of the cultivar 'Bolero' has previously been determined to 10.1 μg/g of fresh weight (*19*). These concentration differences are likely to be caused by variations in soil type, climate, nitrogen fertilization, etc.

In the present study, falcarindiol was the most abundant polyacetylene in peeled carrot roots in agreement with previous studies (*5*, *20*, *25*) but in contrast to a recent study of peeled carrots, where falcarinol was found to be the most abundant analyte (*24*). Previous studies have shown that falcarindiol and falcarindiol-3-acetate are primarily found in the carrot peel, but it should be noted that only peeled carrot roots were investigated in the present study.

No significant differences ($p > 0.05$) in the content of polyacetylenes between the three growth systems (C, OA, and OB) were observed. The concentration levels of falcarindiol-3-acetate were significantly lower in year 2 compared to year 1 ($p = 0.003$), while there was no significant difference between the concentrations of falcarinol and falcarindiol in years 1 and 2 ($p = 0.42$ and

0.07, respectively), although the statistical analysis indicated a lower concentration of falcarindiol in year 2 ($p < 0.10$). This suggests the influence of weather on the content of polyacetylenes in carrots.

The comparable content of polyacetylenes in carrots from the conventional and organic growth systems might be explained by the soil type and fertility, which can reduce the impact of different fertilizer application rates. This was substantiated by the observed harvest yields and dry matter contents, which showed no significant effect between growth year or system (**Table 1**). Only the nitrogen content was significantly different, with higher contents in conventionally grown carrots. This is likely to be a consequence of a higher nutrient supply and nutrient availability of the inorganic NPK fertilizer relative to the treatments with organic fertilization. Thus, the content of polyacetylenes might have differed more significantly between systems if larger differences in fertilizer application rates had been applied on less fertile soil. However, it is important to stress that the fertilizer levels applied reflected common farmer practice in modern carrot production on fertile soils.

The root size has previously been shown to affect the concentration of polyacetylenes in carrots (*5*), but the same tendencies were not observed in the present study, although the carrots from the OB system were significantly larger than the other growth systems (**Table 1**). However, the differences in root size were considerable lower in the present study (average root sizes in years 1 and 2 of 90.2, 88.7, and 102 g of fw/carrot in the C, OA, and OB system, respectively) compared to the root sizes investigated by Kidmose et al. (50−100 and > 250 g of fw/carrot) (*5*).

In conclusion, three polyacetylenes (falcarindiol, falcarindiol-3-acetate, and falcarinol) in carrots were identified and analyzed with an optimized and validated ULP extraction method yielding very fast extractions with improved extraction efficiency in comparison to a traditional stirring based method. The ULP method was combined with the optimized HPLC method leading to a significant reduction in run time. The method is selective, sensitive, and precise for analysis of polyacetylenes in carrot roots. The new method was applied for analysis of carrot roots grown either organically or conventionally, but no statistical differences were found in the content of the possible health-promoting polyacetylenes.

ABBREVIATIONS USED

C, crops grown conventionally with the use of inorganic fertilizers and pesticides; FaDOAc, falcarindiol-3-acetate; FaDOH, falcarindiol; FaOH, falcarinol; i-FIT, isotopic pattern fit of the measurement compared to the theoretical; OA, crops grown organically with the application of animal manure; OB, crops grown organically with the use of cover crops as green manure; ULP, ultrasonic liquid processor; UPLC, ultra-performance liquid chromatograph.

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Supporting Information Available: Extraction efficiency (expressed in μg of falcarindiol equiv/g of dry weight) of the methods tested (Figure S1), extraction efficiency (expressed in μg of falcarindiol equiv/g of dry weight) with ULP in relation to extraction time (Figure S2), polyacetylenes present in the carrots (Table S1), and linear range of measurement, LOD and LOQ, precision (expressed as the RSD; $n = 9$), and recovery (%; $n = 9$) of the analyses of polyacetylenes in carrots (Table S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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