Simple and Direct Analysis of Falcarinol and Other Polyacetylenic Oxylipins in Carrots by Reversed-Phase HPLC and Charged Aerosol Detection

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Abstract

Food plants in the *Apiaceae* (formerly *Umbelliferae*) family (e.g., carrots, parsley, and celery) contain a group of bioactive C17-polyacetylene compounds, sometimes referred to as the polyacetylenic oxylipins. These compounds have been shown to be highly toxic toward bacteria and fungi and to exhibit a diverse range of biological activities in mammals, both beneficial (e.g., their cytotoxicity is proposed to reduce the risk of developing cancer) and detrimental (e.g., occupational allergic contact dermatitis). Three such compounds—falcarinol, falcarindiol, and falcarindiol-3-acetate—are natural pesticides produced by carrots in response to fungal diseases, and have recently garnered significant media attention.

Although falcarinols have a distinctive UV spectrum (the consequence of conjugated triple bonds), sensitivity tends to be poor due to the actual number of unsaturated bonds present in their structure. Measurement at 205 nm offers the best sensitivity; however, sample chromatograms tend to be very complicated due to the presence of many other compounds absorbing at this wavelength.

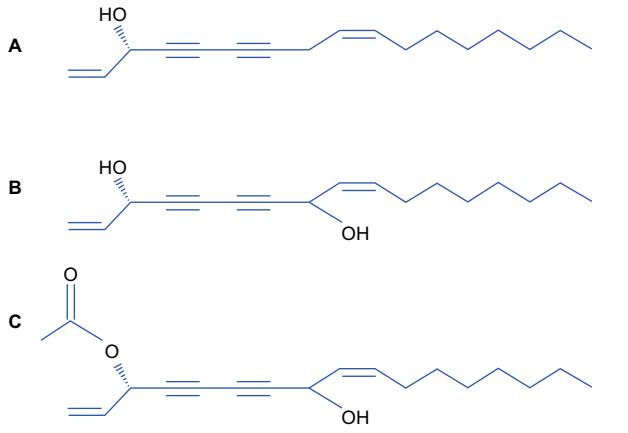
The Thermo Scientific Dionex CAD™ charged aerosol detector is a universal, mass-based detector and offers excellent sensitivity, a wide dynamic range, and the advantage that all nonvolatile analytes produce similar response, independent of chemical structure. Additionally, unlike UV detection, analytes need not possess a chromophore in order to be determined. A simple reversed-phase HPLC-CAD method was developed to rapidly screen for falcarinol, falcarindiol, and falcarindiol-3-acetate. The method was sensitive (LOQ ~5 ng on column) and reproducible, and the analysis was completed in 15 min. Data from fresh baby carrots and Queen Anne's Lace (root, leaf, and flower) are presented.

Introduction

Many useful compounds with pharmacological interests have been found in nature, including the anticancer agent (paclitaxel) from the yew plant, the immunosuppressants (FK506 and cyclosporine) from a sea coral, and a soil fungus, as well as many steroids, opiates, and amphetamines.

In carrots and other *Apiaceae* plant species, a number of oxylipin or polyacetylene compounds are found, including falcarinol, falcarindiol, and falcarindiol-3-acetate (shown in Figure 1). They are produced by plants, primarily as an antifungal compound, protecting roots from licorice fungus as well as bacteria. Interestingly, recent studies have found that these compounds have an inhibitory affect on colorectal and leukemia cancer cells in rats.[1,2]

FIGURE 1. Structures of A) falcarinol, B) falcarindiol, and C) falcarindiol-3-acetate.



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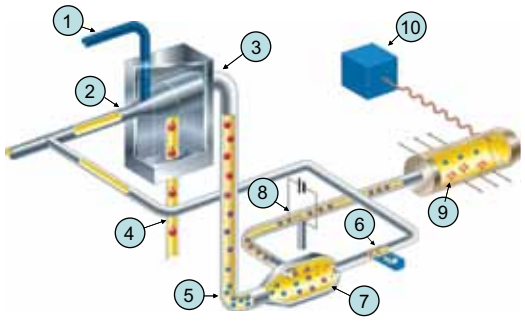
Oxylipins typically occur at low levels. Several kilograms of carrots are needed to obtain 40–60 mg quantities of these oxylipins, which are then purified through one or more preparatory HPLC processes. Thus, highly sensitive analyses are required. Falcarinol and its related polyacetylene impurities require low-wavelength UV detection for sensitive and accurate determinations by HPLC. This often can be problematic because mobile phase modifiers and flow irregularities can interfere with baselines, and extinction coefficients for analytes at these wavelengths vary widely.

Falcarinol, falcarindiol, and falcarindiol-3-acetate were determined in a number of samples, including parsnip, carrots (baby carrots, mature carrots, and baby food), celery, and parsley, using an HPLC method with the mass-sensitive Thermo Scientific Dionex Corona™ *ultra*™ CAD charged aerosol detector.

The Dionex CAD is a sensitive, mass-based detector especially well suited for the determination of any nonvolatile analyte, independent of chemical characteristics. As shown in Figure 2, the detector uses nebulization to create aerosol droplets. The mobile phase evaporates in the drying tube, leaving analyte particles that become charged in the mixing chamber. The charge is then measured by a highly sensitive electrometer, providing reproducible, ng-level sensitivity. This technology has greater sensitivity and precision than evaporative light scattering (ELS) and refractive index (RI), and it is simpler and less expensive to operate than a mass spectrometer (MS).

FIGURE 2. Schematic of Dionex Corona CAD flow paths.



1. Liquid eluent enters from the HPLC system
2. Pneumatic nebulization occurs
3. Small droplets enter the drying tube
4. Large drops exit to drain
5. Dried particles enter the mixing chamber
6. Gas stream passes over corona needle
7. Charged gas collides with particles and transfers charge
8. High mobility species are removed
9. Remaining charged particles measured
10. Signal is transferred to chromatographic data software

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Methods

HPLC Method Conditions

HPLC System:	Thermo Scientific Dionex UltiMate™ 3000 HPLC
Column:	Thermo Scientific Acclaim™ RSLC 120 C18, 2.2 µm, 150 × 2.1 mm
Mobile Phase A:	Methanol/water/acetic acid (500:500:4)
Mobile Phase B:	Acetone/methanol/tetrahydrofuran/ acetic acid (500:375:125:4)

Gradient:

Time (min)	*%B*
0	0
1	40
10	60
15	100
15	0
20	0

Flow Rate:	0.65 mL/min
Inj. Volume:	5 µL at 10 °C
Column Temp.:	50 °C
Detector:	Dionex Corona *ultra*
Nebulizer Heater:	30 °C
Filter:	High
Sample Solvent:	Methanol/chloroform (2:1)

Sample Preparation

An amount of material was vortex mixed in methanol/ chloroform (2:1) for 1 h at room temperature. Centrifugation was used to remove the solid particles, and the extracts were injected directly for analysis.

Results and Discussion

Falcarinol, falcarindiol, and falcarindiol-3-acetate were dissolved in methanol/chloroform (2:1) to 100 µg/mL. Serial dilutions were prepared, each at 50% relative concentrations to final values below 0.8 µg/mL (4 ng on column [o.c.]), and analyzed in triplicate. Chromatograms of each are shown in Figures 3, 4, and 5. From these injections, it was possible to determine the area-percent purity of each of the standards and compare each against the HPLC-UV analysis that was obtained at a wavelength of 205 nm.

FIGURE 3. Chromatogram of falcarindiol, 500 ng o.c.

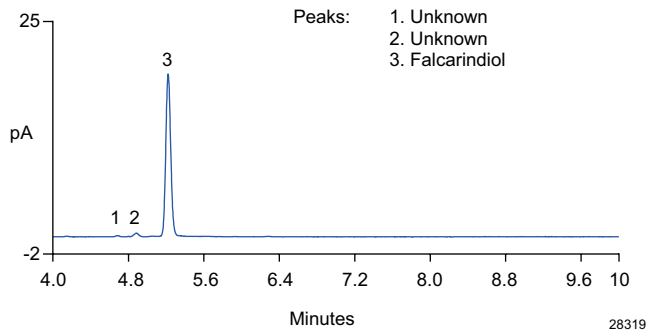


FIGURE 4. Chromatogram of falcarindiol-3-acetate, 500 ng o.c.

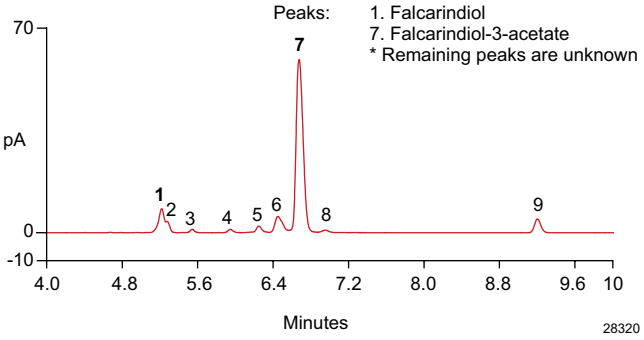
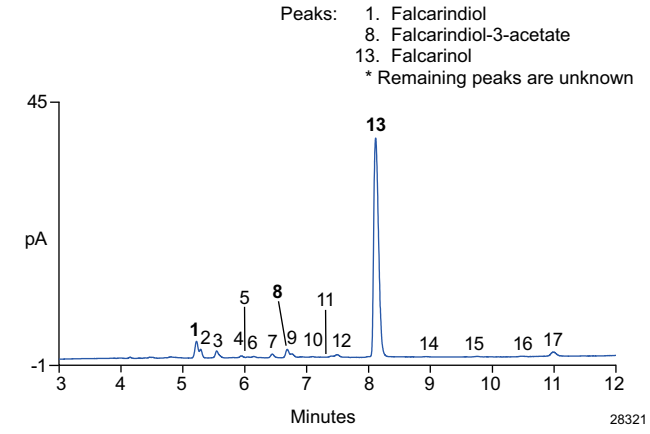


FIGURE 5. Chromatogram of falcarinol, 500 ng o.c.



The comparison of the purity values showed a significant difference between the two analyses, as shown in Table 1. The peak-area percent values for purity were 10 to 20% lower for the HPLC-CAD values, compared to those analyzed by HPLC-UV. Falcarindiol—at approximately 100% purity—was the purest of the three analytes tested by HPLC-UV. With HPLC-CAD, however, the purity was 90.1%, showing a greater amount of impurities than the HPLC-UV method.

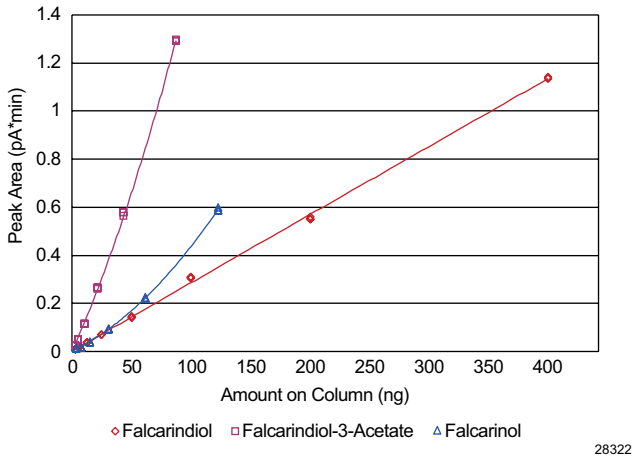
Table 1. Percent Area Values by Detector,UV at 205 nm and CAD

Analyte	LC-UV$_{205}$ (AREA-%)	LC-CAD (AREA-%)
Falcarindiol	~100.0	90.5
Falcarindiol-3-Acetate	91.5	71.2
Falcarinol	97.4	81.4

Falcarindiol-3-acetate showed the greatest amount of impurities, with the greatest difference between the two modes of detection: 91.5 area % by HPLC-UV and 71.2 area % by HPLC-CAD. This standard also appeared to contain a peak with the same retention time as facarindiol, so this area was proportionately subtracted from the falcarindiol peak when they were analyzed as a combined standard solution. The differences in response factors between UV and CAD for the various impurities were significant, which has major implications for concentration-dependent or purity-dependent evaluations of these compounds.

Calibration curves were plotted and fit with second-order polynomials, shown in Figure 6, with CAD area % used as a mass purity to correct o.c. amounts and coanalyte peak areas removed. Each amount was analyzed in triplicate. All correlation coefficients (r^2) were greater than 0.999 for plots ranging from 3 to the highest area of each component needed for these determinations. Precision values for RSD were less than 4% for all amounts >7 ng o.c.

FIGURE 6. Calibration curves for falcarinol, falcarindiol, and falcarindiol-3-acetate (n=3).



Method sensitivity was determined at the lower o.c. amount of 3.9 ng (at 100% purity), using signal-to-noise ratios equal to 3 for the limit of detection (LOD) and equal to 10 for the limit of quantitation (LOQ). Falcarindiol-3-acetate yielded the greatest sensitivity, with low ng o.c. amounts for LOD, as shown in Table 2. These sensitivities are similar to another HPLC UV method[4] using 205 nm, but as shown above, other impurities may not absorb at this wavelength.

Table 2. LOD and LOQ Values

Analyte	LOD (ng o.c.)	LOQ(ng o.c.)
Falcarindiol	2	5
Falcarindiol-3-Acetate	1	2
Falcarinol	3	11

Samples were prepared and analyzed directly without any derivatization. All samples were finely diced and extracted with 5× grams of material per mL of solvent and vortex mixing. Centrifugation was used to remove particulates, and the clean supernatant was directly injected. Example HPLC-CAD chromatograms from parsnip skin, parsnip core, and parsley leaf are shown in Figures 7, 8, and 9, respectively.

The last sample showed the most complex chromatogram, but the three oxylipins were still easily determined. All analyte quantities were calculated from peak areas found in each sample, and the analytical results are presented in Table 3. The amount of polyacetylenes in carrots was consistent with literature values.[3] A higher proportion of falcarindiol was found in the carrot perideam than in the phloem (core). Values for the falcarindiol-3-acetate were significantly different from literature values, possibly a result of different solvent used for extraction, or differences in standard purity/amount used for calibration, which is a frequent issue with biologically based standards. From earlier work using a fused-core C8 HPLC column, Queen Anne's Lace (Daucus carota, wild carrot) was also processed and analyzed. The total amounts of falcarindiol, falcarindiol-3-acetate, and falcarinol were 425 µg/g fresh weight (FW), 327 µg/g FW, and 278 µg/g FW, for the flower, root, and leaf, respectively. None of these analytes were detected in the baby carrots.

FIGURE 7. Parsnip skin: 0.692 g in 3.46 mL methanol/ chloroform (2:1).

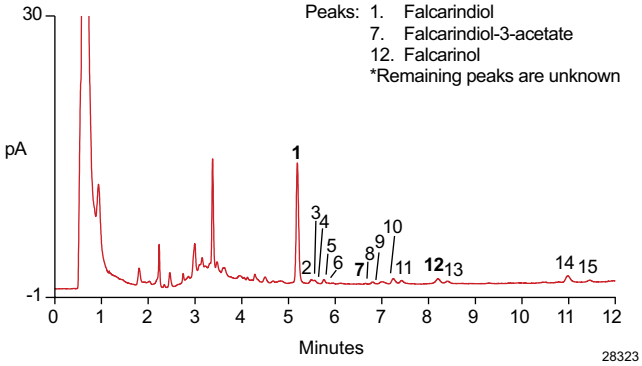


FIGURE 8. Parsnip core: 2.055 g in 10.28 mL methanol/ chloroform (2:1).

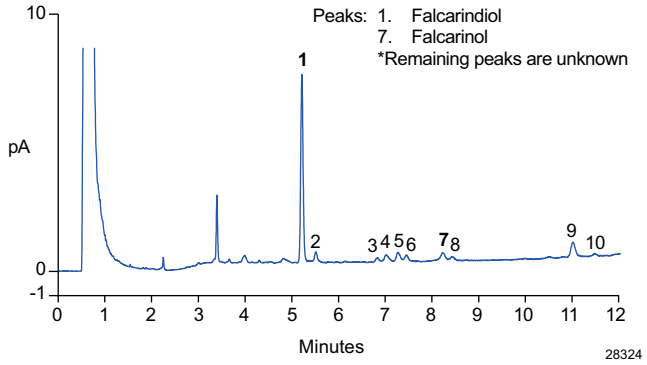
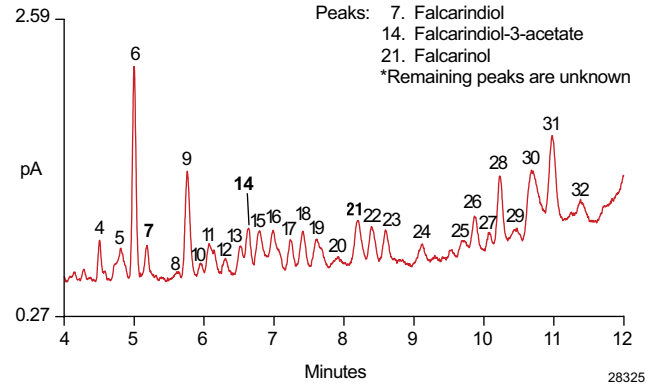


FIGURE 9. Parsley: 0.2083 g in 1.04 mL methanol/ chloroform (2:1).



Conclusion

This study demonstrates use of the Dionex Corona CAD charged aerosol detector in determining the amounts of three poorly chromophoric analytes in sample extracts. The polyacetylene standards used for calibration were also analyzed by HPLC using the Dionex CAD charged aerosol detector. Differences in peak area purity of these standards between UV and CAD were found, with CAD showing greater amounts of impurities than UV. This was attributed to the low and varying extinction coefficients of these oxylipins and the lack of response of the impurities by UV.

The HPLC-CAD method provides quantifiable results with calibration curves that are highly correlated. The method is also precise, with low percent RSD values across the concentrations analyzed and sensitivities to the single digit ng level.

Vegetable samples were extracted and directly analyzed, demonstrating the method's selectivity for oxylipins contained in complex sample matrices (e.g., parsley leaf in Figure 9). Values obtained demonstrated relevance to literature values and practical experience: parsnips are generally perceived to be more bitter than carrots, which is reflected in the greater amounts of these compounds present in parsnips than in carrots.

The Dionex Corona charged aerosol detector provides the sensitivity, reproducibility, and dynamic range that is required to analyze these compounds in complex matrices.

Table 3. HPLC-CAD Analytical Results of Samples, mg of Analyte Per FW, Grams

Sample	Falcarindiol (µg/g FW)	Falcarindiol-3-acetate (µg/g FW)	Falcarinol (µg/g FW)
Carrot, skin	144	0.7*	11
Carrot, core	36	0.7*	8*
Parsnip, skin	289	0.5*	25
Parsnip,core	175	nd	16
Celery, stalk	2*	nd	4*
Celery, leaves	10	nd	6*
Parsley, stems	6	nd	6*
Parsley,leaf	7	2.9	18
Carrots, baby food	36	0.7*	6*

*Analytes detected, but amounts are below LOQ.

References

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