Profiling of Citrus Oils and Determination of Furocoumarins in Citrus Oils Using the Agilent 1290 Infinity 2D-LC Solution

Application Note

Food Testing & Agriculture

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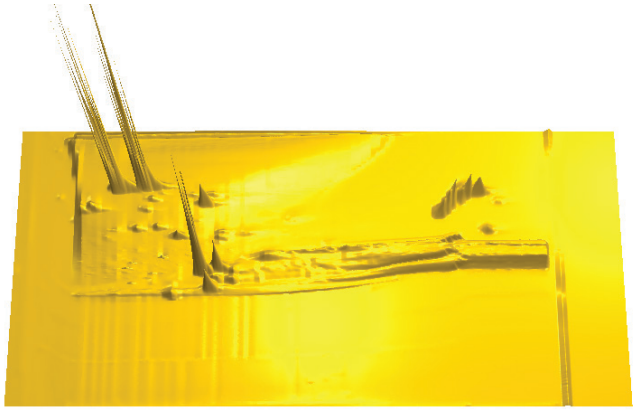
Abstract

Comprehensive two-dimensional liquid chromatography (LC×LC) using the Agilent 1290 Infinity 2D-LC Solution with DAD and MS detection is applied for the analysis of citrus oils. The developed method uses normal phase liquid chromatography in the first dimension and reversed-phase liquid chromatography in the second dimension. This combination provides good orthogonality and is valuable for profiling various citrus oils. The method also enables the detection of (potentially) carcinogenic furocoumarins in citrus oil samples.



Verified for Agilent
1290 Infinity II LC

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Introduction

Citrus oils are applied in numerous consumer products such as cosmetics, sun protection and bronzing formulations, food flavoring, and so forth. They are generally extracted from fresh citrus peel by mechanical cold-pressing. These oils contain significant amounts of psoralens (lemon oils), methoxylated flavonones, and flavones (orange oils). A specific species of psoralens, namely furocoumarins, have been identified as photomutagenic and photocarcinogenic products. The International Agency for Research on Cancer (IARC) has classified 5-methoxypsoralen, (5-MOP, bergapten) and 8-methoxypsoralen, (8-MOP, xanthotoxin) when combined with UV radiation as group 2A (probably carcinogenic to humans) and as group 1 (carcinogenic to humans) risk carcinogens, respectively. On that basis, limits have been defined for the presence of psoralens in cosmetics. The Commission Directive 95/34/DC of 1995 states that furocoumarines should be below 1 mg/kg (1 ppm) in sun protection and in bronzing products[1,2].

It is important to detect furocoumarins in the essential oils used in cosmetics. Certain furocoumarins, such as the phototoxic 5-MOP, are not always detected and the relative amounts can differ according to type, origin, and season of citrus fruit harvesting[3]. The analysis of such compounds in citrus oils can be done by (U)HPLC. The results have been described by the authors in Agilent Technologies Application Note 5990-4033EN[4]. Chromatograms of such samples can be complex, and there is only limited certainty that a small peak for a target compound (such as a furocoumarin) will be separated from other sample constituents in a wide range of sample types (citrus species, origin, blending, and so forth). Frérot and Decorzant reported that the difficulty of furocoumarin analysis in citrus oils lies in the presence of numerous products such as coumarins and flavonoids and that even the best HPLC methods will not always avoid coelution[3]. In order to increase the separation probability significantly, a radical increase in peak capacity is required. This can be accomplished by using comprehensive two-dimensional LC (LC×LC). The Agilent 1290 Infinity 2D-LC Solution was used to compare various citrus oils and to detect furocoumarins in such samples. A combination of normal phase LC (NPLC) in the first dimension and reversed-phase LC (RPLC) in the second dimension resulted in good orthogonality. Combining these two very different separation modes is challenging due to mobile phase incompatibility issues. However, the combination of NPLC/RPLC has previously been demonstrated for lemon and orange oil analysis[5,6].

This Application Note presents data on the comparison of various citrus oils and the detection of furocoumarins in these oils. On-line mass spectroscopic detection was also used with 2D-LC for confirmation of the identity. A fully automated system with easy method setup, gradient development, and complete software integration was used.

Experimental

Solutions and samples

All solvents were HPLC gradient grade from Biosolve B.V. (Valkenswaard, the Netherlands). The furocoumarin standards were from Sigma-Aldrich (Bornem, Belgium). Figure 1 shows the structure of the standards. Stock solutions of the standards were prepared in ethanol and further diluted in ethanol/hexane 50/50 or oil. The lemon oil samples were from Italy and Argentina

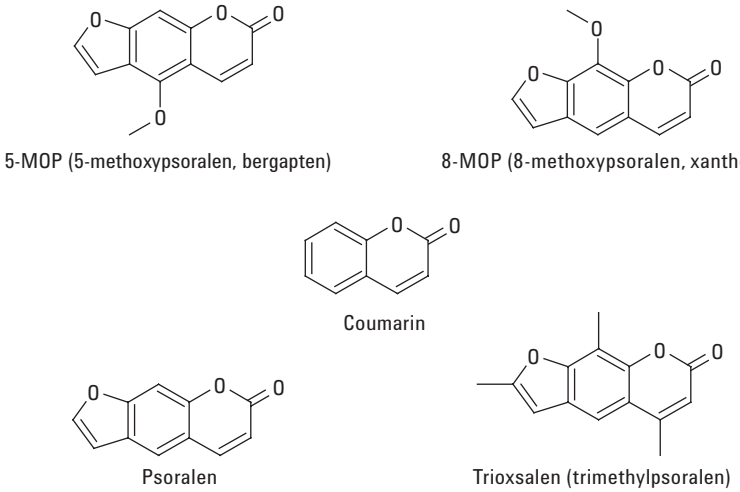

Figure 1. Structures of furocoumarin standards.

Instrumentation

An Agilent 1290 Infinity 2D-LC Solution was used. The configuration is shown below:

Instrument	Part number
Agilent 1290 Infinity Binary pump (1st dimension)	G4220A
Agilent 1290 Infinity Binary pump (2nd dimension)	G4220A
Agilent 1290 Infinity Autosampler	G4226A
Agilent 1290 Infinity Autosampler Thermostat	G1330A
Agilent 1290 Infinity Thermostatted Column Compartment	G1316C
Agilent 1290 Infinity Diode Array Detector with standard flow cell	G4212A
Agilent 1290 Infinity Valve Drive	G1170A
2 position/4-port duo valve for 2D-LC	G4236A
Agilent Single Quadrupole LC/MSD with APCI source	G6130B

and the orange oil sample were supplied by Quest International (Naarden, The Netherlands). All other oil samples were purchased locally.

Software

- Agilent OpenLAB CDS Chemstation revision C.01.04 with 2D-LC add-on software

- GC Image LC/LC Edition Software for 2D-LC data analysis (GC Image, LLC., Lincoln, NE, USA)

Method

1st Dimension	
Column	Agilent ZORBAX RX-SIL, 1.0 × 150 mm, 3.5 µm (custom packed)
Solvent A	Hexane/Ethylacetate 95/5 (v/v)
Solvent B	Ethylacetate
Flow rate	35 µL/min
Gradient	0 to 35 minutes: 0 to 40 % B
	35 to 36 minutes: 40 to 70 % B
	36 to 60 minutes: 70 to 90 % B
Post-time	10 minutes at 0 % B
Temperature	25 °C
2nd Dimension	
Column	Agilent ZORBAX Eclipse Plus C18 RRHD, 3.0 × 50 mm, 1.8 µm (p/n 959757-302)
Solvent A	Water
Solvent B	Acetonitrile
Flow rate	2.2 mL/min
Idle flow rate	0.3 mL/min
Gradient	0 to 0.38 minutes: 0 to 100 % B, repeated
Temperature	40 °C
Modulation	
Modulation on	2.5 to 52 minutes
Loops	Two 20-µL loops, cocurrent configuration
Modulation time	0.50 minutes
Injection	
Volume	Pure oil: 0.4 µL, oil mix: 0.8 µL
Sample temperature	15 °C
Needle wash	6 seconds flushport (ethylacetate/isopropanol/acetone)
Detection DAD	
Wavelength	Signal 315/4 nm, Reference 500/50 nm
Data rate	80 Hz
Detection MSD	
Ionization mode	APCI
Source settings	
Drying gas flow	7 L/min
Drying gas temperature	340 °C
Nebulizer pressure	55 psi
Vaporizer temperature	410 °C
Capillary voltage	3,000 V (pos and neg mode)
Corona current	4 µA (pos mode) and 15 µA (neg mode)
Detection mode	FastScan, 150–700 *m/z*, Fragmentor 90 V

Results and Discussion

Oil profiling

Various oil samples were analyzed with this setup. Using UV detection at 315 nm, several coumarins and psoralens (described in the literature) can be detected and identified. Figure 3 shows the plot for the analysis of a lemon/orange oil mix. The mixture contains various psoralens and analogues (lemon oils, compounds 1–14) and methoxylated flavonones and flavones (orange oils). The latter are considerably more polar compared to the lemon oil compounds and need a stronger mobile phase to elute from the normal-phase column. Therefore, a fast gradient to 70 % ethylacetate is applied from 35 to 36 minutes. As a consequence, the second dimension gradient needs to start with a 100 % aqueous mobile phase throughout the run. This focusses the loop content on the reversed-phase column. Starting with an organic portion in the second dimension gradient leads to some phase mixing and has adverse effect on the peak width in the reversed-phase separation.

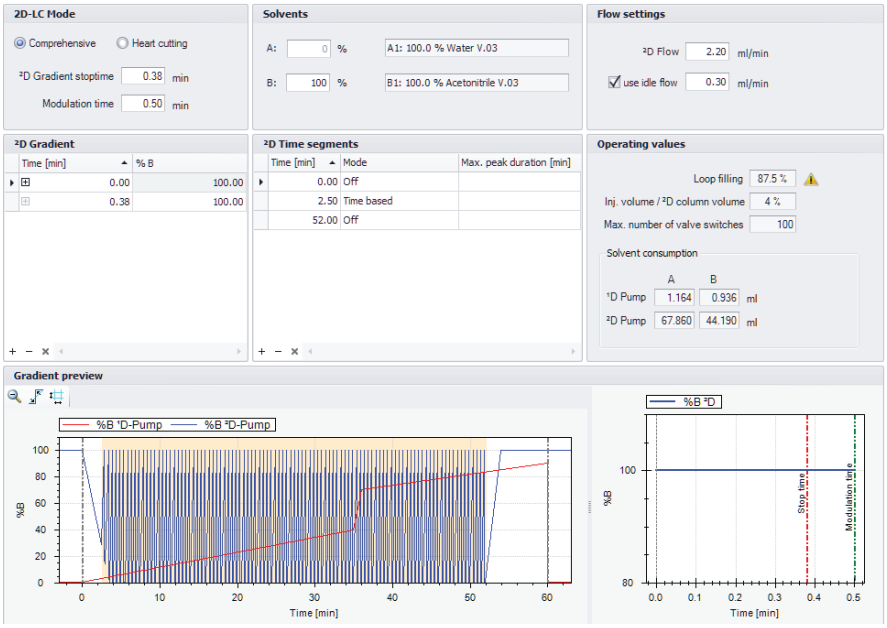

Figure 2. Screenshot of the 2D-LC method (OpenLAB CDS ChemStation)

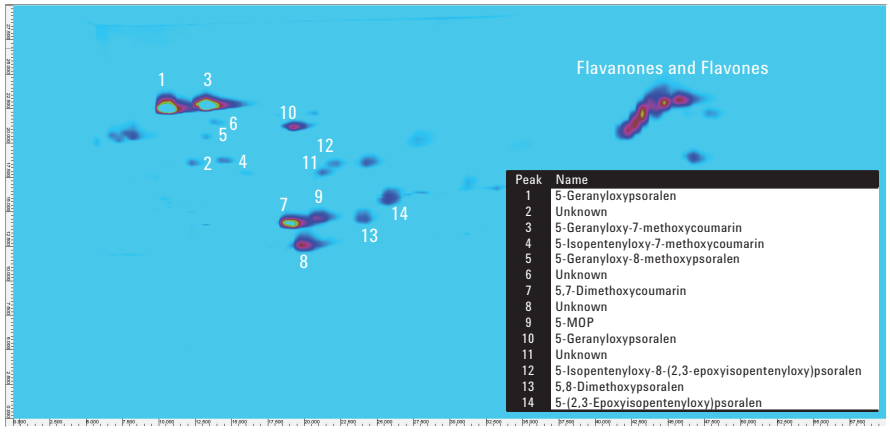

Figure 3. Analysis of a lemon/orange oil mix with the described system. Detection: DAD 315 nm.

One bergamot oil and three different lemon oil samples were analyzed with the same method. From the results in Figure 4 it is clear that the psoralen composition is substantially different between the samples. Interestingly, one of the known carcinogens, 5-MOP Compound 9), is detected in some samples. This is in accordance with the literature and our previous findings in one-dimensional UHPLC[4]. The bergamot oil contains more 5-MOP and 5-geranyloxypsoralen (bergamottin, Compound 1) than the other citrus oils.

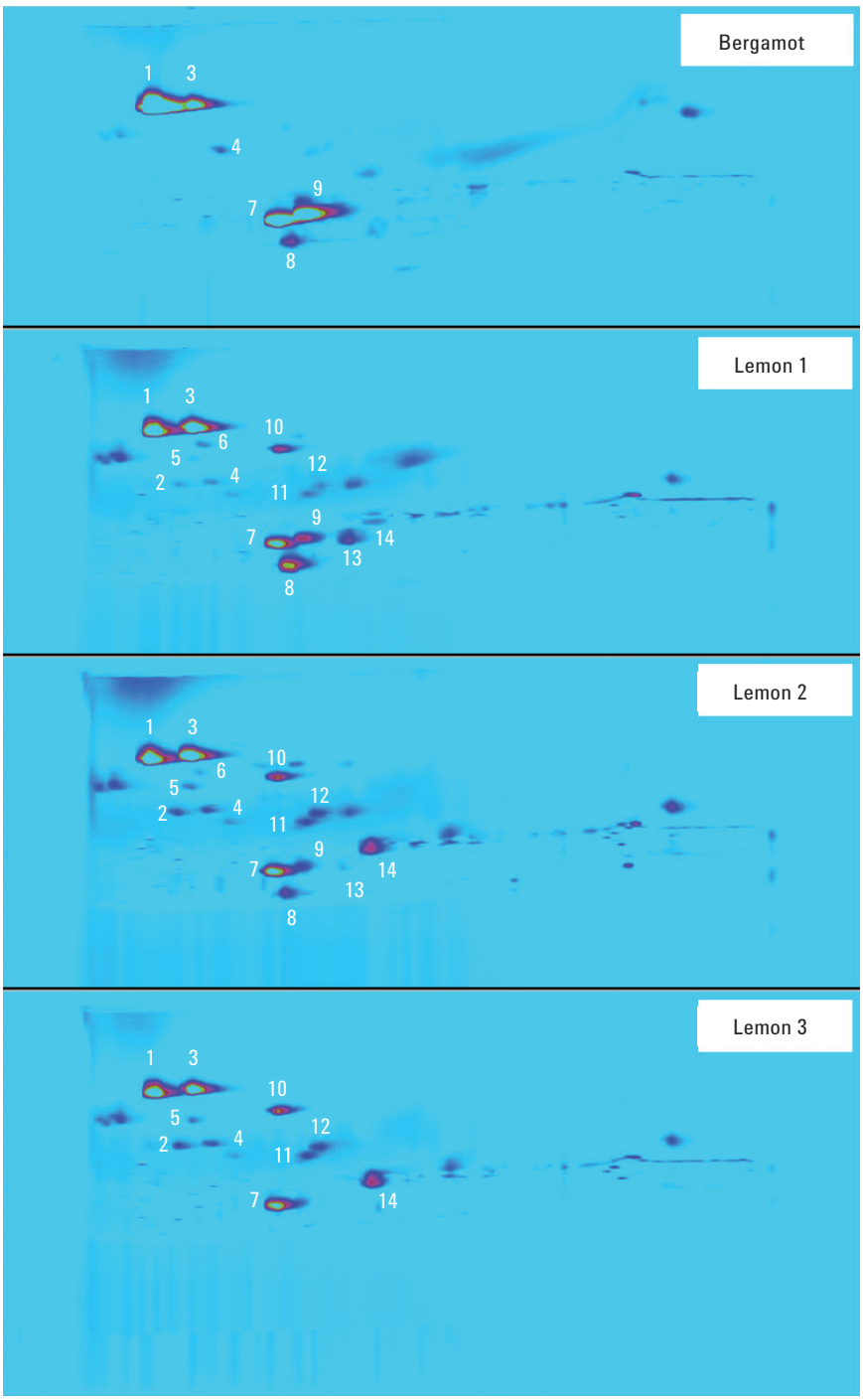

Figure 4. Analysis of a various citrus oil samples. Detection: DAD 315 nm. (Identification: see Figure 3)

Furocoumarins

To investigate the presence of the target compounds, the five standards were diluted in ethanol/hexane 50/50 or oil and analyzed. Because of increased detectability for some compounds at 254 nm, this wavelength was preferred above the 315 nm used for citrus oil profiling. Figure 5 shows the plots of a standard mixture and a spiked mixed oil sample. It is clear from the contour plot that there are coelutions between target compounds itself and between targets and matrix constituents in both of the single dimensions. When projecting spots 15 and 16 to the X-axis (1D, normal phase), they will not be separated from each other. Projection to the Y-axis (2D, reversed-phase) shows a significant difference in retention, and thus separation. Compounds 17 and 18 are separated from each other but coelute with other target and matrix compounds in the first dimension. The orthogonality with the second dimension results in separation for these compounds.

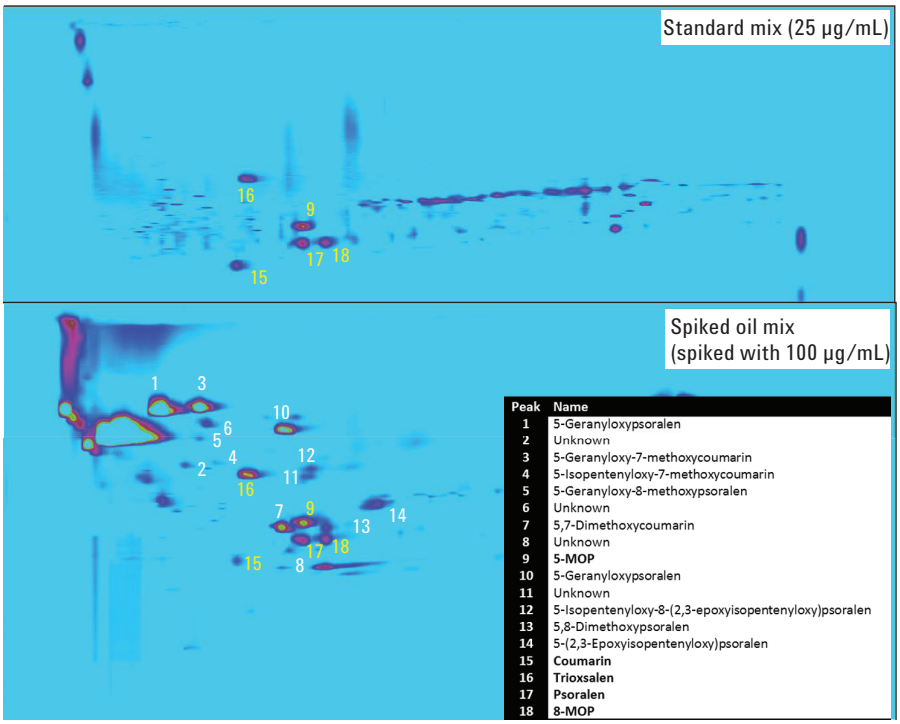

Figure 5. Comparison of the results for a standard solution and a spiked oil mix sample. The target furocoumarins are shown in yellow. Detection: DAD 254 nm.

Since 5-MOP was the only target detected in the final samples, quantitative analysis was focused on this compound. After calibrating from 10 to 100 µg/mL, the 5-MOP content in the oil samples was determined to be ca. 656, 138, 56, and 51 µg/mL oil for bergamot oil, lemon oil 1, lemon oil 2, and oil mix, respectively. No 5-MOP could be detected in lemon oil 3. The identity of the 5-MOP was confirmed with comparing UV and mass spectra in samples with the spectra obtained with standard solutions. To perform MS detection, the effluent from the D2 column was split by a T-piece. The Agilent 1290 Infinity DAD was connected to the T-piece by a 70 mm × 0.12 mm id stainless steel capillary, and the inlet of the 6130 Single Quadrupole MSD APCI source was connected to the other outlet of the T-piece by a 340 mm × 75 µm stainless steel capillary. The 75 µm capillary acts as a restriction and routes the largest part of the flow towards the DAD. The spectra are shown in Figure 6.

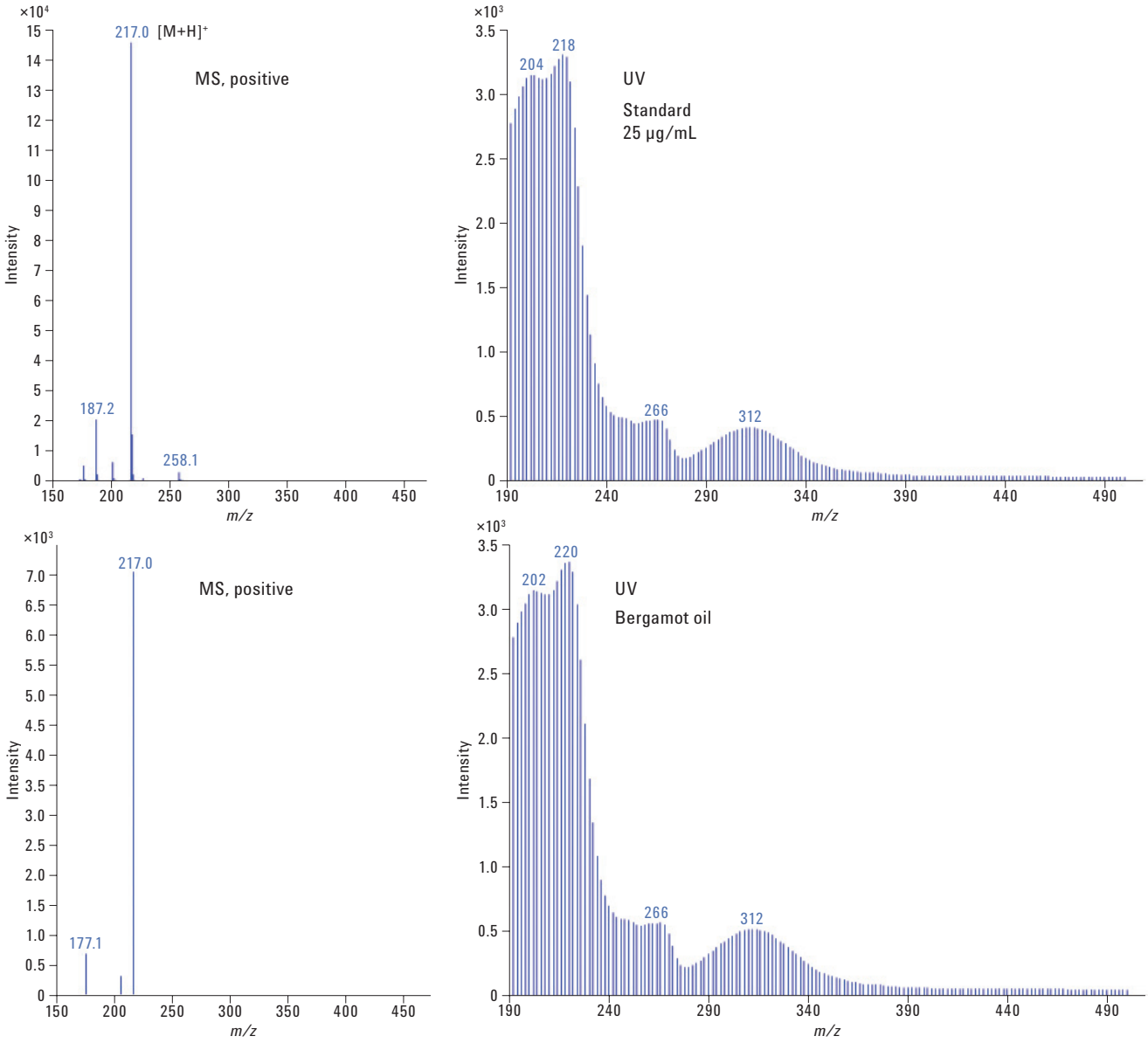

Figure 6. UV and MS spectra (positive ionization) taken from the GC image plots for 5-MOP in a standard solution and the bergamot oil sample.

Conclusion

The Agilent 1290 Infinity 2D-LC Solution with DAD was successfully applied for the analysis of various citrus oils. Different lemon oils were compared and the method proved to be useful for characterization of the oils regarding their psoralen, methoxylated flavonone, and flavone content.

The system was hyphenated with MS to further assist compound identification. The carcinogenic compound 5-MOP was detected and quantified in three out of four citrus oils.

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