Screening and Identification of Natural Products in Citrus Oil

Non-Target Analysis using SWATH® Acquisition, Compound Identification Using the NIST Library

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Overview

Analysis of natural products in citrus oils by non-targeted acquisition techniques is described using the SCIEX X500R QTOF system and MS/MS library matching against the NIST mass spectral library for compound identification. The polymethoxylated flavonoids of the non-volatile fraction of the citrus oils are of interest in describing the screening workflow. An important feature of the method was that the same acquisition run was used to monitor both the natural products and screen for pesticide suspects. Several hundred natural products were identified using the non-target peak finding processing. Using a targeted screening list, 14 compounds were detected in the 3 citrus oils (Valencia orange, lemon and grapefruit).

Introduction

Citrus oils are widely used in the food and cosmetic industries for their flavour and fragrance properties. In addition, citrus oils are increasing employed as therapeutic agents due to their apparent antibacterial, antifungal and anticarcinogenic activities.[1] The non-volatile fraction of citrus oils contains coumarins, furocoumarins, and polymethoxylated flavonoids (PMFs). Specifically, the PMFs have shown anti-inflammatory and anti-carcinogenic effects.[2] Therefore, identification of these natural products in citrus oils is important to assess therapeutic content. In addition, it is important to screen for pesticide compounds to ensure safety.

Non-target acquisition (NTA) techniques are beneficial for chemical screening since they allow for wide compound coverage without sacrificing scan time. QTOF instruments, such as the X500R, are ideal for NTA because they collect high resolution accurate mass data on both the precursors and their fragments at cycle times which are satisfactory for HPLC and UPLC peak widths. SWATH® Acquisition is a NTA technique that is advantageous over traditional information dependent acquisition (IDA) techniques. SWATH Acquisition collects MS/MS spectra on all precursor compounds, whereas IDA is limited because only the most intense precursors trigger MS/MS. Therefore, SWATH Acquisition will typically result in greater unknown compound identifications.

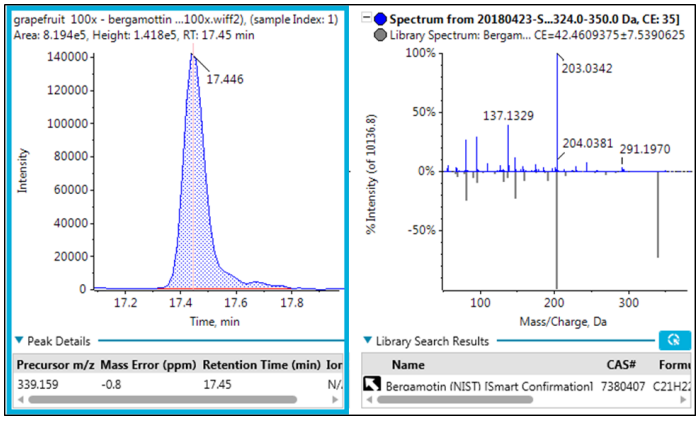

Figure 1. Identification of Bergamottin in Grapefruit Peel Oil Extract. Left panel shows precursor XIC (extraction width = 20 mDa), right panel shows experimental MS/MS pattern (top) and NIST MS/MS library match for bergamottin (bottom).

Accurate identification of unknown compounds is greatly strengthened by matching experimental MS/MS fragmentation patterns with published library databases. The NIST MS/MS database contains fragmentation spectra for ~14,000 compounds and has recently been converted to a SCIEX LibraryView™ format for easy searching. For example, Figure 1 shows the NIST library match for bergamottin in grapefruit peel oil.

Key Workflow Benefits

- Flexibility of QTOF workflow and data acquisition means endogenous natural products and trace pesticide residues can be analyzed in the same sample injection
- The addition of the NIST MS/MS library significantly improves the ability for unknown compound identification.
- SWATH Acquisition ensures that MS/MS information was collected on all precursors, resulting in MS/MS information available for peak ID

Experimental

Sample Preparation: Three cold-pressed citrus oils (Valencia orange, lemon and red grapefruit) were received from a commercial producer. For analysis, the oils were diluted 100-fold in acetonitrile and thoroughly vortexed.

Chromatography: The SCIEX ExionLC™ AD LC system was utilized and chromatographic separation was achieved under gradient conditions using the Phenomenex® Luna Omega Polar C18 column (100 Å, 2.1 x 100 mm, 1.7 µm particle size). The mobile phases were water (0.1% formic acid, 5mM ammonium formate) and acetonitrile (0.1% formic acid) with a flow rate of 200 µL/min. The column oven was maintained at 40ºC and the injection volume was 1 µL.

Table 1. LC Gradient Program. Using a flow rate of 200 µL/min, injection volume = 1 µL.

Step	Time (min)	A (%)	B (%)
0	0.0	95	5
1	20.0	5	95
2	25.0	5	95
3	25.1	95	5
End	32.0		

Mass Spectrometry: The SCIEX X500R QTOF system with Turbo V™ source using the electrospray ionization (ESI) probe war run in positive ion mode. Source and gas conditions are presented in Table 2. Data were collected using SWATH Acquisition with variable window widths. The SWATH Acquisition windows were chosen to minimize the precursor Q1 density during the pesticde screening since the natural product molecular weights were unknown at time of analysis (Table 3). Using variable SWATH Acquisition windows help reduce the complexity of MS/MS fragmenention spectrum thus improving library matching and improvng unknown identification. TOF MS (scan range: 100-1500 Da) parameters were DP = 80 V, CE = 10 V and accumulation time = 0.10 sec. The TOF MS/MS (scan range 50-1000 Da) parameters were DP = 80 V, CE = 35 V, CES = +/-15 V and accumulation time = 0.05 sec. The collision energy spread (CES) ensures that a comphrensive MS/MS pattern is collected.

Table 2: Source, Gas and Temperature Conditions.

Parameter	Value
Curtain Gas (CUR)	30 psi
Collision Gas (CAD)	12
IonSpray Voltage (IS)	*5500 V*
Temperature (TEM)	*550ºC*
Nebulizer Gas (GS1)	*50 psi*
Heater Gas (GS2)	*50 psi*

Data Processing: All data processing was performed within the SCIEX OS software 1.5. Data were initially processed using the "non-target peaks" module in Analytics. This module uses the peak finding algorithm to extract features from the TOF MS TIC and then assigns the applicable MS/MS fragmentation spectrum to that precursor. The peak detection sensitivity was set to the middle level since the natural products were expected to be in high concentration. To aid in the identification of extracted features, experimental MS/MS fragmentation spectra were searched against the National Institute of Technology (NIST) MS/MS Spectral Library. This NIST database is beneficial in this workflow because it contains a large number of natural products. Data review efficiency was optimized by utilizing the qualitative rule "traffic lights" which filtered out features which did not have corresponding NIST library hits.

In addition, a list of 15 PMFs was used for targeted screening (Table 4). In the processing method, the user-defined chemical formulae and adduct/charge were used to calculate the exact mass (extracted mass width = 20 mDa). Compounds were positively identified if the mass error was <5 ppm, experimental isotope ratio was within 20% of the theoretical value, and MS/MS library matching score for fit was >70.

Table 3: Variable SWATH Acquisition Windows.

Start	End	Width
100	175	75
174	200	26
199	225	26
224	250	26
249	275	26
274	300	26
299	325	26
324	350	26
349	375	26
374	400	26
399	425	26
424	450	26
449	475	26
474	500	26
488	525	26
524	1000	476

Results

Pesticides

A components list of 274 pesticides was built and retention times were determined from the authentic standards. Overall, 19 unique pesticides were detected in the 3 citrus oil samples (10 pesticides per sample). Analytes were considered positively detected if they met the following criteria: mass error <5 ppm, isotope pattern error <20%, retention time error <2.5% and library fit score >70.

An example detection for azoxystrobin in lemon peel oil is shown in Figure 2. The XIC shows that the retention time difference was 0.2%, precursor mass error was -0.7 ppm, isotope ratio difference was 6.1% and the library fit score was 100.

These results demonstrate that both the natural products and pesticides can be analyzed in the same acquisition method, highlighting the flexibility of the X500R QTOF system.

Table 4: Components List for Targets PMF Screening.

Parameter	Formula	Adduct	Precursor Mass (Da)
5,7-Dimethoxycoumarin	*C11H10O4*	*[M+H]+*	*207.0652*
Bergapten	*C12H8O4*	*[M+H]+*	*217.0495*
Osthol	*C15H16O3*	*[M+H]+*	*245.1172*
Meranzin	*C15H16O4*	*[M+H]+*	*261.1121*
Isomeranzin	*C15H16O4*	*[M+H]+*	*261.1121*
Naringenin	*C15H12O5*	*[M+H]+*	*273.0758*
Aurapten	*C19H22O3*	*[M+H]+*	*299.1642*
Bergamottin I	*C21H22O4*	*[M+H]+*	*339.1591*
Bergamottin II	*C21H22O4*	*[M+H]+*	*339.1591*
Tetra-O-Methylscutellarein	*C19H18O6*	*[M+H]+*	*343.1176*
Epoxybergamottin	*C21H22O5*	*[M+H]+*	*355.154*
Tangeretin	*C20H20O7*	*[M+H]+*	*373.1282*
Sinensetin	*C20H20O7*	*[M+H]+*	*373.1282*
Nobiletin	*C21H22O8*	*[M+H]+*	*403.1387*
3,5,6,7,8,3',4'-Heptamethoxyflavone	*C22H24O9*	*[M+H]+*	*433.1493*

Natural Products: Non-Target Peak Processing

The non-target peak finding algorithm extracted ~1500 features between the 3 citrus peel samples. However, not all features yielded good chromatographic peaks and strong MS/MS spectra. Manually reviewing each chromatogram would be tedious and time consuming. Therefore, the qualitative rule “traffic lights” was used to quickly filter the data. Since no prior knowledge of the compounds were known, the results table was filtered to only show positive library matches (i.e. library fit score >70).

After filtering the data to display only positive library hits, the data was sorted by either area count, or the area ratio of comparison (ratio of sample area count to blank area count) to focus on the dominant natural products.

After filtering and sorting the data, many natural flavonoids (e.g. 5,6,7,3',4'-pentamethoxyflavone, Figure 3), PMFs (e.g. osthol) and pesticides (e.g. pyraclostrobin) were identified through MS/MS spectral matching with the NIST library. The diversity of identified compound classes demonstrates extensive coverage of the NIST mass spectral library as well as the broad application of the chromatography and SWATH Acquisition method.

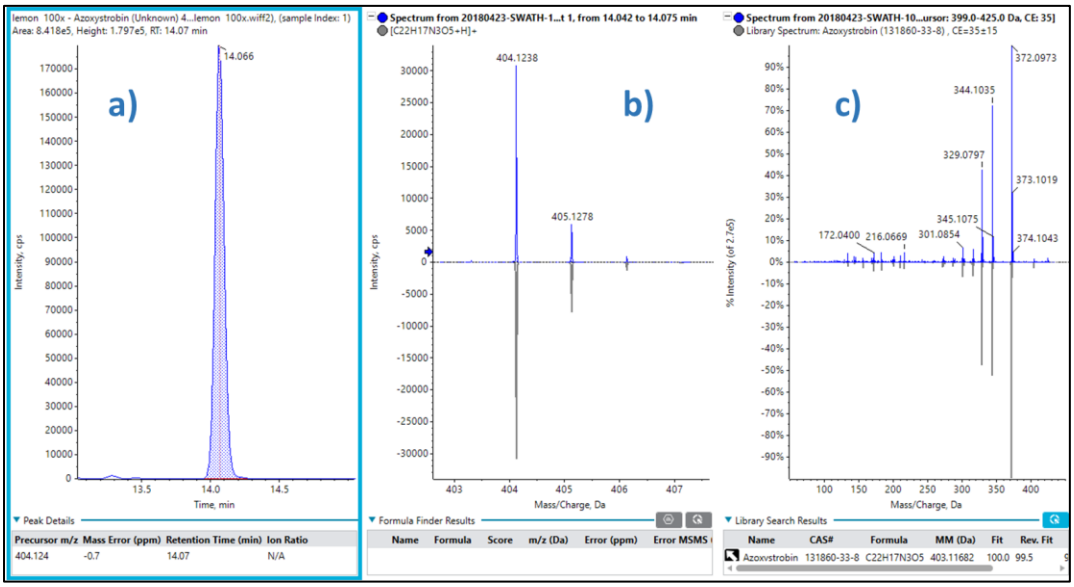

Figure 2. Peak Review Pane for the Detection of Azoxystrobin in Lemon Peel Oil. Panels show: TOF MS XIC (a), MS spectrum (b) and MS/MS spectrum (c) showing comparison between experimental (top) and library match (bottom).

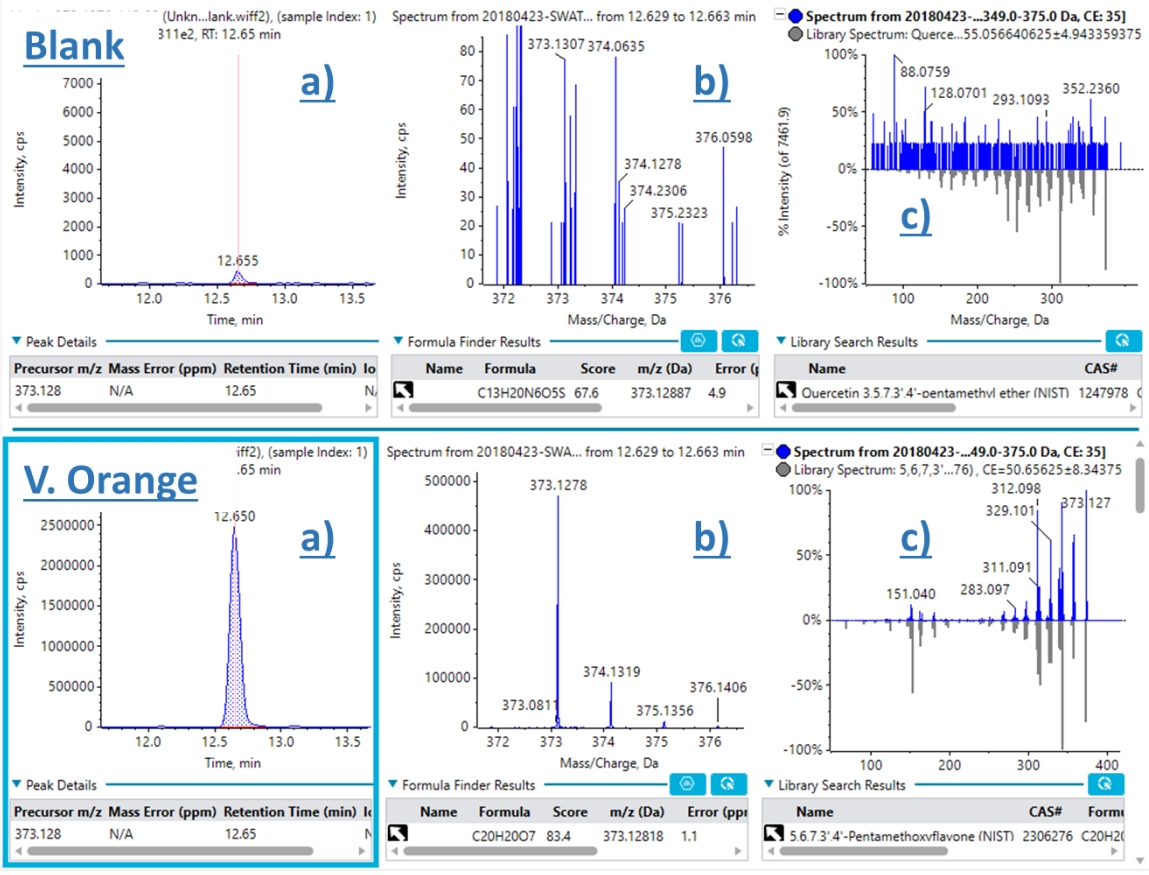

Figure 3. Peak Review Pane. Easy visualization of m/z 373.1278 (RT = 12.65 min) showing blank (top) and Valencia Orange Peel oil (bottom). Panels show the a) TOF MS XIC b) MS spectrum and c) MS/MS spectrum with NIST library match for 5,6,7,3',4'-pentamethoxyflavone.

Natural Products: Targeted Screening

The non-target workflow demonstrates the ability of the X500R with SWATH Acquisition and MS/MS library matching to broadly identify natural products such as the PMFs. Therefore, the sample data was reprocessed with a targeted components list to focus on 15 specific PMFs of interest. For the targeted screening workflow, the precursor XIC mass is calculated from the chemical formula and adduct/charge state. MS/MS library matching was used to confirm the compound identity. Since retention time was unknown, the "retention time mode" in the components table was set to "find 5 peaks" (Figure 6). This feature extracts the 5 peaks, at each XIC mass, that possess "acceptable" mass errors (i.e. <5 ppm) – the retention time was confirmed by comparing to the MS/MS library match.

All 15 targeted PMFs were detected in at least 1 citrus oil sample through "acceptable" mass error (<5 ppm), isotope ratio difference and positive MS/MS library hit (Figure 1, Table 5). Grapefruit had the most individual PMFs detected with all compounds observed with the exception of epoxybegamottin.

It was not possible to distinguish between meranzin & isomeranzin, as well as between tangeretin & sinensetin since these pairs are structural isomers (i.e. identical accurate masses) and have similar MS/MS fragmentation spectra. For example, tangeretin and sinesetin differ by the relative position of 1 methoxy group. Therefore, authentic standards will be needed to confirm these compounds by their retention times.

Two peaks were present in the XIC for bergamottin and both peaks showed positive MS/MS library matches for bergamottin. Thus, it is possible that these are also structural isomers and additional experiments will be needed to elucidate the definitive structure. Further, 3,5,6,7,8,3',4'-hexamethoxyflavone did not have a positive MS/MS library match but did have acceptable mass error and isotope ratio difference for all samples.

Table 5: Individual PMFs Detected in Citrus Oil.

Compound	RT (min)	Valencia Orange	Lemon	Grapefruit	
5,7-Dimethoxycoumarin	*11.7*		✔	✔	
Bergapten	*11.9*			✔	
Osthol	*14.9*	✔		✔	
Meranzin	*12.0, 12.2*	✔	✔	✔	
Isomeranzin	*12.0, 12.2*	✔	✔	✔	
Naringenin	*10.6*			✔	
Aurapten	*17.7*			✔	
Bergamottin I	*17.5*		✔	✔	
Bergamottin II	*18.3*		✔	✔	
Tetra-O-Methylscutellarein	*13.4*	✔	✔	✔	
Epoxybergamottin	*15.5*	✔	✔		
Tangeretin	*13.9, 12.6*	✔	✔	✔	
Sinensetin	*13.9, 12.6*	✔	✔	✔	
Nobiletin	*13.3*	✔		✔	
3,5,6,7,8,3',4'-Heptamethoxyflavone	*13.6*	✔*	✔*	✔*	

Conclusions

A comprehensive suite of pesticides and natural products were identified in citrus oils through non-target SWATH Acquisition with MS/MS fragmentation spectral matching against the NIST library. Non-target data processing demonstrated the potential to detect hundreds of natural products whereas a targeted screening list was used to focus on 15 individual PMFs.

A unique feature of this workflow is that both chemical classes – pesticides and natural products – were analyzed in the same injection, thus greatly simplifying the analysis.

References

1. Dosoky, N.S; W.N. Setzer. Biological Activities and Safety of Citrus spp. Essential Oils. (2018) *Int. J. Mol. Sci.* **19(7)**, 1966.
2. Fan, H.; Wu, Q.; Simon, J.E.; Lou, S. -N.; C.-T Ho. Authenticity analysis of citrus essential oils by HPLC-UV-MS on oxygenated heterocyclic components. (2015) *J. Food Drug Anal.*, **23(1)**, 30-39.
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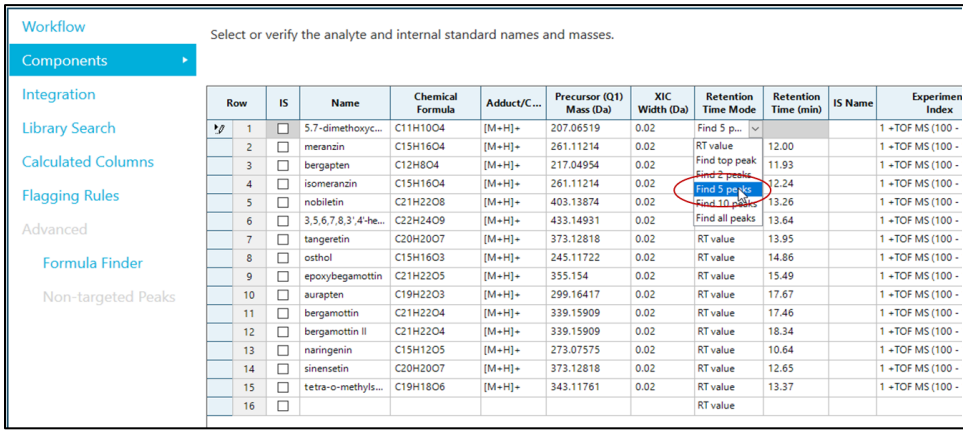

Row	IS	Name	Chemical Formula	Adduct/C...	Precursor (Q1) Mass (Da)	XIC Width (Da)	Retention Time Mode	Retention Time (min)	IS Name	Experimen Index
1	☐	5.7-dimethoxyc...	C11H10O4	[M+H]+	207.06519	0.02	Find 5 p...			1 +TOF MS (100 -
2	☐	meranzin	C15H16O4	[M+H]+	261.11214	0.02	RT value	12.00		1 +TOF MS (100 -
3	☐	bergapten	C12H8O4	[M+H]+	217.04954	0.02		11.93		1 +TOF MS (100 -
4	☐	isomeranzin	C15H16O4	[M+H]+	261.11214	0.02		12.24		1 +TOF MS (100 -
5	☐	nobiletin	C21H22O8	[M+H]+	403.13874	0.02		13.26		1 +TOF MS (100 -
6	☐	3,5,6,7,8,3',4'-he...	C22H24O9	[M+H]+	433.14931	0.02		13.64		1 +TOF MS (100 -
7	☐	tangeretin	C20H20O7	[M+H]+	373.12818	0.02	RT value	13.95		1 +TOF MS (100 -
8	☐	osthol	C15H16O3	[M+H]+	245.11722	0.02	RT value	14.86		1 +TOF MS (100 -
9	☐	epoxybegamottin	C21H22O5	[M+H]+	355.154	0.02	RT value	15.49		1 +TOF MS (100 -
10	☐	aurapten	C19H22O3	[M+H]+	299.16417	0.02	RT value	17.67		1 +TOF MS (100 -
11	☐	bergamottin	C21H22O4	[M+H]+	339.15909	0.02	RT value	17.46		1 +TOF MS (100 -
12	☐	bergamottin II	C21H22O4	[M+H]+	339.15909	0.02	RT value	18.34		1 +TOF MS (100 -
13	☐	naringenin	C15H12O5	[M+H]+	273.07575	0.02	RT value	10.64		1 +TOF MS (100 -
14	☐	sinensetin	C20H20O7	[M+H]+	373.12818	0.02	RT value	12.65		1 +TOF MS (100 -
15	☐	tetra-o-methyls...	C19H18O6	[M+H]+	343.11761	0.02	RT value	13.37		1 +TOF MS (100 -
16	☐						RT value			

Figure 6. SCIEX OS Analytics Module Showing the Retention Time Mode Feature of "Find 5 Peaks".

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