Quick and Sensitive Analysis of Multiclass Veterinary Drug Residues in Meat, Plasma, and Milk on a Q Exactive Focus LC-MS System

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Application Note 614

Key Words

Q Exactive Focus, Orbitrap, veterinary drugs, HRAM quantitation, HRAM screening, vDIA, unknown screening, retrospective data analysis

Goal

To describe a new method for veterinary drug analysis, showing how the variable data-independent acquisition (vDIA) workflow achieves high sensitivity and selectivity, providing a complete high-quality data record of the measured sample. Quantitative analysis of the acquired data in combination with non-targeted and unknown screening is shown.

Introduction

The analysis of veterinary drugs in animal products is usually a time-consuming process with respect to both sample preparation and mass spectrometric analysis. The quantitative analysis of multiclass veterinary drug residues from animal products—including meat, milk, and plasma—often requires multiple sample injections in order to achieve optimal conditions for individual classes of compounds. This includes multiple chromatographic methods for different compound classes, as well as separate mass spectrometric methods, each specifically directed to small groups of compounds. The data obtained only contains information on the targeted compounds and is not suitable for any retrospective analysis on additional analytes of interest.

Here, a new method utilizing ultrafast chromatography with the Thermo Scientific™ Q Exactive Focus™ benchtop Orbitrap™ mass spectrometer is described. It consists of a short generic chromatographic method and a new mass spectrometric method called variable data-independent acquisition (vDIA). The advantages of this approach are short overall analysis time, superior selectivity, and high sensitivity. This robust method provides data with suitable options for additional targeted and non-targeted screening. The vDIA approach has been developed and is utilized here for generation of calibration curves and analyses of samples for known and unknown targets. vDIA can use multiple MS/MS isolation windows with widths from 50 Da up to 800 Da. Typically, smaller windows are used for lower mass regions to increase dynamic range and

Figure 1. Q Exactive Focus benchtop Orbitrap mass spectrometer.

therefore sensitivity, while larger windows cover higher mass regions to improve the duty cycle. In a typical acquisition setup, shown here, five MS/MS isolation windows were set to cover the entire mass range of the preceding full scan while maintaining speed of MS/MS analysis for the fast chromatography.

Forty-four multi-class veterinary drug residues listed in Table 1, were analyzed in extracts of muscle, kidney, milk, and plasma using a single standardized chromatographic and mass spectrometric method. For absolute quantification, standard samples with known concentrations of all 44 drug residues covering eight calibration points (from 100 pg/mL (ppt) to 500 ng/mL (ppb)) were prepared. For evaluation of the method, spiked matrix samples (muscle and kidney for antibiotics, milk for avermectins and plasma for nitroimidazoles) were analyzed by high-resolution, accurate-mass (HRAM) LC-MS/MS.

vDIA method is not available in the United States of America.

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Table 1. List of components used for analysis with limits of quantitation (LOQ).

Compound	LOQ (ppb)	Compound	LOQ (ppb)
Abamectin*	5.0	Marbofloxacine	5.0
Amoxicillin	1.0	Metronidazole	0.5
Ampicillin	0.5	Metronidazole-OH	0.5
Cefalexin	0.5	Moxidectin	0.5
Cefalonium	0.5	Nafcillin	0.5
Cefaperazone	1.0	Oxacillin	0.1
Cefapirim	0.1	Penicillin G	0.5
Cefquinome	5.0	Penicillin V	0.5
Chlorotetracycline	1.0	Ronidazol	0.5
Ciprofloxacin	0.5	Sarafloxacine	0.5
Cloxacillin	0.1	Sulfadiazine	0.1
Danofloxacin	5.0	Sulfadimethoxin	0.5
Dapsone	0.5	Sulfadimidin/Sulfamethazine	0.1
Difloxacin	0.5	Sulfadoxin	0.5
Dimetridazol	5.0	Sulfamerazin	0.1
Doramectin*	10.0	Sulfamethoxazole	0.5
Doxycyclin	0.5	Sulfamethoxypyridazine	0.1
Enrofloxacin	1.0	Sulfathiazole	0.5
Eprinomectin	5.0	Tetracycline	0.5
Erythromycine	1.0	Thiamphenicol	0.5
Flumequine	1.0	Trimethoprim	0.1
Ipronidazol-OH	0.5	Tylosine	1.0

Experimental

Liquid Chromatography Method

A generic LC method was used for all samples:

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 Rapid Separation LC (RSLC)
Column	Thermo Scientific™ Accucore™ C18 aQ 100 x 2.1 mm, 2.6 µm particle size (p/n 17326-102130)
Mobile phase A	Fisher Chemical water + 0.1% formic acid
Mobile phase B	Fisher Chemical acetonitrile + 0.1% formic acid
Gradient	6 min gradient from 5% B to 95% B
Flow rate	300 µL/min
Total chromatographic cycle	15 min

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Mass Spectrometry Method

A generic full-scan method with wide-isolation variable data-independent acquisition (FS-vDIA) was used for all samples:

Instrumentation	Q Exactive Focus MS system
Full Scan	
Resolution setting	70,000 (FWHM) at *m/z* 200
Mass range (*m/z*)	100–1000
vDIA	
Resolution setting	17,500 (FWHM) at *m/z* 200
Isolation windows (*m/z*)	100–205, 195–305, 295–405, 395–505, 495–1000
Spray voltage	4.4 kV
Sheath gas	30.0 arb.
Aux gas	5.0 arb.
Capillary temp.	250 °C
Heater temp.	300 °C
RF-lens level	50
HCD collision energy	35 eV

Data Processing

Data processing was performed using Thermo Scientific™ TraceFinder™ software version 3.2. For generation of extracted ion chromatograms, an extraction window of 5 ppm was used. For non-targeted screening, a built-in component and fragment *m/z* values database was used, consisting of 450 components.

Analytes were quantified based on full scan information (quasi molecular ions). Additionally, one to five fragment ions were used for identity confirmation, according to EU regulatory requirements (EC/657/2002), achieving linear calibration curves over the ranges described above.

Results and Discussion

The vDIA approach described bridges the gap between full-scan data-dependent MS^2 (FS-ddMS^2) experiments and full range fragmentation scan modes such as all-ion fragmentation (AIF). As Figure 2 shows, this is a combination of a full scan with several wide range isolation MS^2 scans. In this setup, the isolation widths of the MS^2 windows vary between 100 Da and 500 Da and together cover the entire mass range of the preceding full scan.

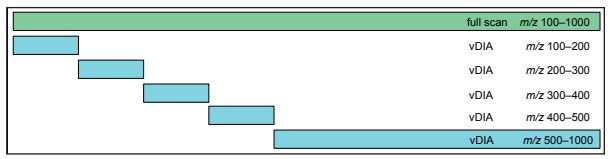

Figure 2. Setup of a typical FS-vDIA experiment.

FS-ddMS^2 experiments, where MS^2 scans are performed on targets of interest (present on an inclusion list) upon their detection in the full scan, are known to be very selective and sensitive with respect to the fragment ion information obtained. Retrospective FS-ddMS^2 data analysis for additional compounds of interest, however, is limited to full-scan quantitation by accurate mass without confirmation of identity by MS/MS.

Full-range fragmentation experiments like AIF, where fragments from all species present in the full scan are detected in a single MS^2 scan, have the advantage of collecting all possible full scan and MS^2 information for the sample. Thus, they are fully suitable for retrospective data analysis. Dynamic range, selectivity, and achieved detection limits, however, are limited as the number of ions fragmented per species is lower due to the combined nature of the analyses.

The vDIA approach, where fragments from wide isolation windows covering the entire mass range are detected in multiple MS^2 scans, maintains very high levels of sensitivity and selectivity while keeping a full digital record of the sample. Therefore, it is fully suitable for retrospective data analysis. Table 1 shows the observed limits of detection (LOQs) in a dilution series for full scan quantitation with fragment ion confirmation. LOQ is defined here as the lowest concentration level on which a component could be confirmed with at least one fragment ion.

For accurate quantification and confirmation of identity, it is crucial that the fragment ions used for confirmation are clearly resolved in time and mass, so that the confirmation is not affected by elevated background or interfering peaks. Figure 3 shows examples of how the overlay of the extracted ion chromatograms of the confirming fragment ions (right panel) match with the quantifier precursor ion (left panel), acquired in vDIA scan mode and automatically processed in TraceFinder software. All confirming ions were free of any interference and co-eluted with the quantifier, providing unambiguous confirmation that is essential, particularly for complex matrices. Since the vDIA mode generates full elution profiles for all fragment ions, the chromatographic peaks of the fragments can be integrated and this way the fragment ion ratio can be calculated with high confidence and used for confirmation as well.

vDIA method is not available in the United States of America.

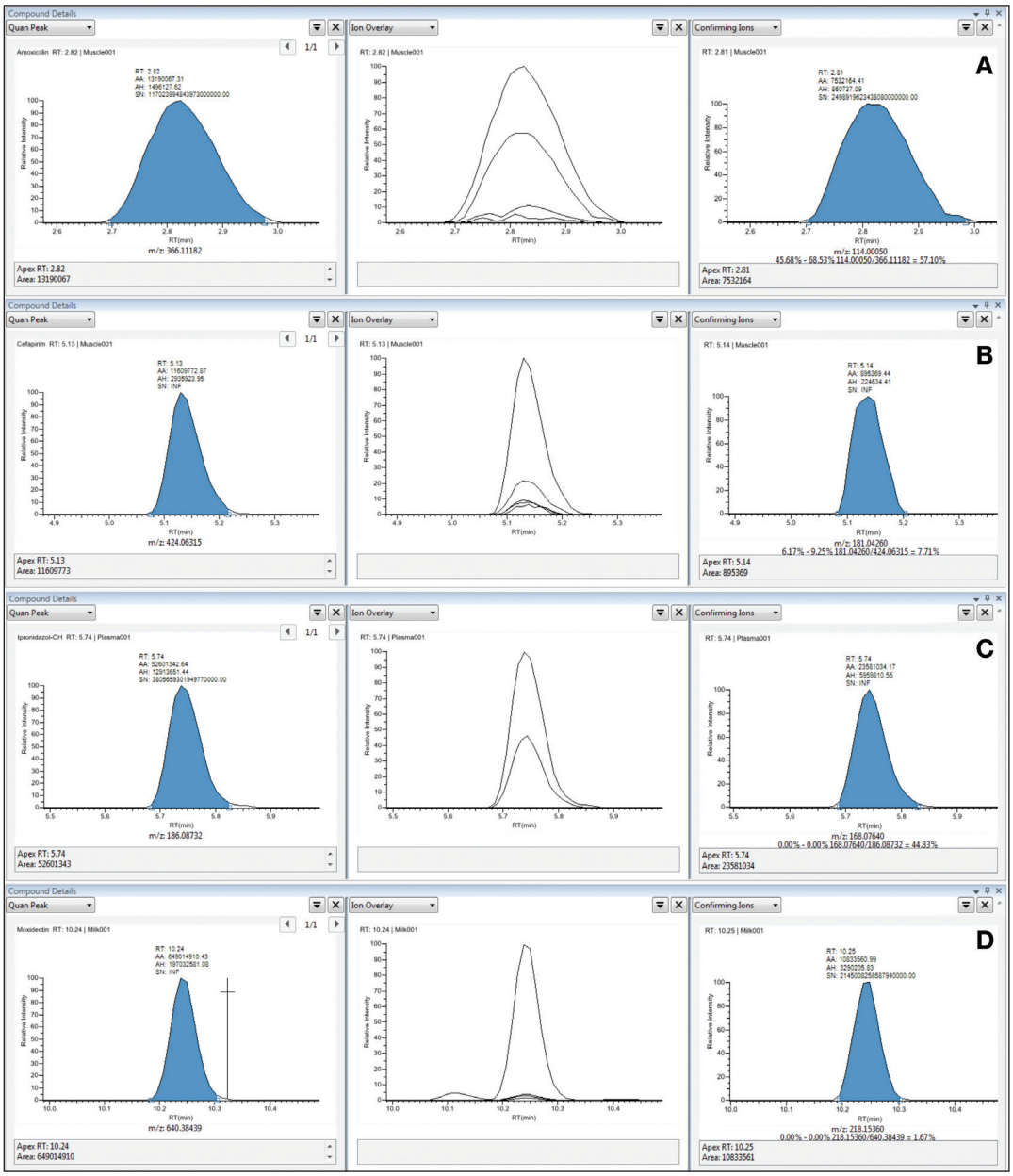

Figure 3. Selectivity of selected components in matrix; A: ampicillin in pig muscle at 5 ppb; B: sulfadiazin in pig kidney at 5 ppb; C: ronidazol in pig plasma at 1 ppb; D: moxidectin in cow milk at 1 ppb.

Figure 4 shows an example of linear dynamic range for selected compounds using the vDIA approach. The calibration range spanned from 0.5 ppb to 500 ppb and linearity in all cases was better than $R^2 = 0.99$.

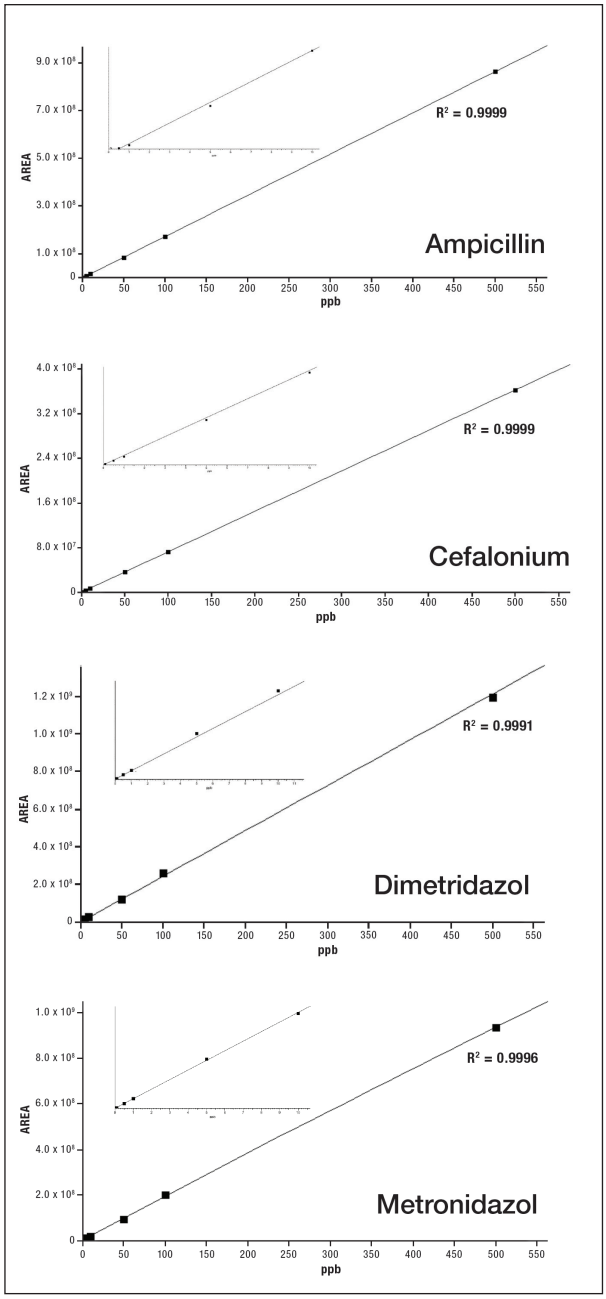

Figure 4. Linearity of selected compounds.

In the cases of abamectin and doramectin, limited stability of the quasi-molecular ions was observed and the sensitivity in the full scan detection was therefore limited. This instability may be due to the use of average parameters for this generic method, suitable for a wide panel of drugs. In these few cases, the vDIA approach allows quantitation using several fragment ions of abamectin and doramectin, permitting quantitation and identity confirmation down to much lower levels than the quasi-molecular ions in the full scan.

After quantitation curves were established, all components were quantified in the spiked matrices at low levels. Antibiotics were spiked into muscle and kidney matrix at levels of 5 ppb, while abamectins were spiked into milk at a level of 1.0 ppb and nitroimidazoles were spiked into plasma at a level of 1.0 ppb.

The TraceFinder processing software provides component detection with high selectivity (by means of the narrow extraction window of 5 ppm) in the full scan with identification according to retention time. In addition to this, TraceFinder software offers three automated options for confirmation of identity. The first uses isotopic information of the precursor detected in the full scan. Figure 5 shows an overlay of the detected spectrum (in red) of ciprofloxacin and the theoretical isotopic distribution (in blue). With a resolution setting of 70,000, even in the complex matrix of muscle extract at a concentration of 5 ppb, the isotopic distribution match is free from interference and gives an unambiguous confirmation.

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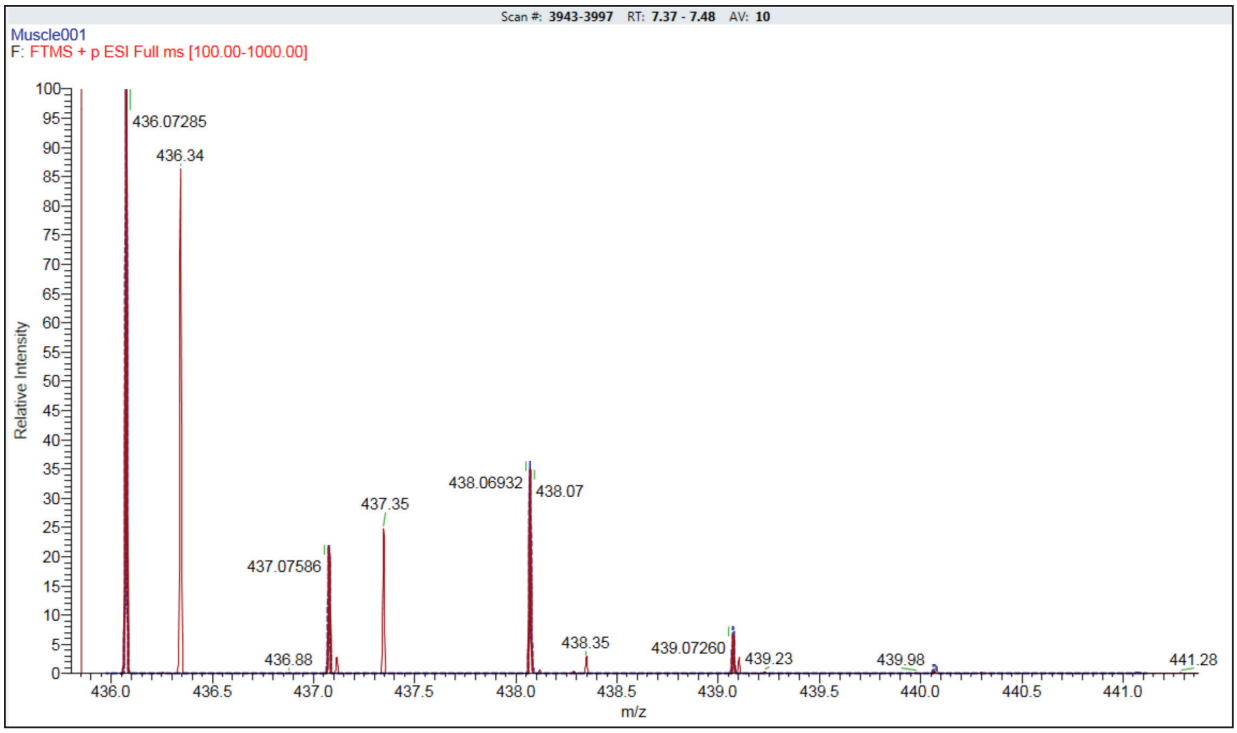

Figure 5. Isotope pattern match for the example of ciprofloxacin. The detected spectrum is shown in red, and the theoretical isotopic distribution is shown in blue.

Confirmation of identity can be performed using the MS/MS data by detecting known fragment ions. The following results were achieved for a muscle tissue sample at a spike level of 0.5 ppb. Figure 6 shows the result of the fragment ion confirmation in vDIA mode. As the vDIA spectra are generated using a wide isolation window, fragment ions from multiple precursors can be detected. The high-resolution, accurate-mass nature of Orbitrap MS/MS detection allows selective identification of the fragment ions combined with RT profile matching giving confident identification.

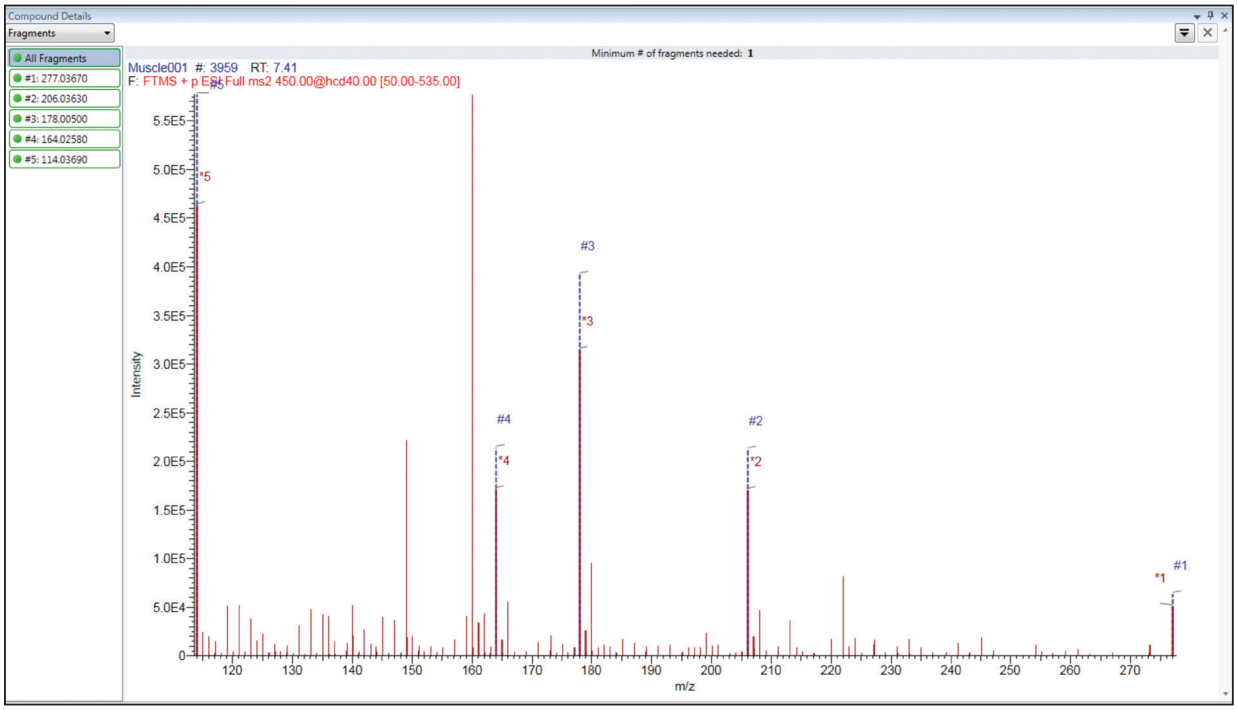

Figure 6. Fragment match for the example of ciprofloxacin. The experimental spectrum is shown in red, the confirmation masses are shown in blue.

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Most routine targeted methods only cover a limited number of analytes. The ability to reanalyze the sample for a suspected drug post-MS analysis becomes very beneficial for saving instrument/lab time and sample. To demonstrate retrospective analysis of the data files, a wide-range screening approach was performed on vDIA data files of analytes spiked in muscle tissue samples. A non-targeted screen with a 1500 component built-in database was conducted, providing several strong matches to additional components present in the sample. Figure 7 shows the example of cortisol (hydrocortisone), confirmed by isotopic pattern match, fragment search, and library match.

Conclusion

The Q Exactive Focus MS in combination with an UltiMate 3000 HPLC was used to create a variable data-independent acquisition method for the detection and quantification of 44 multiclass veterinary drug residues. This novel method proved to have the required LOD sensitivity, and ability to confirm identity using retention time, accurate *m/z*, isotopic ratio, and fragment ions to exceed the EU regulatory requirements (EC/657/2002). The vDIA approach extends confirmation options for non-targeted and unknown screening approaches, while maintaining a high level of sensitivity and selectivity. All data processing was accomplished with the easy-to-use TraceFinder software both for identification and all stages of identity confirmation. vDIA accomplishes the goal of being an accurate and sensitive analysis for the detection of non-targeted compounds (i.e. those from the 44 compound panel) and also for the screening of unknown compounds post-analysis.

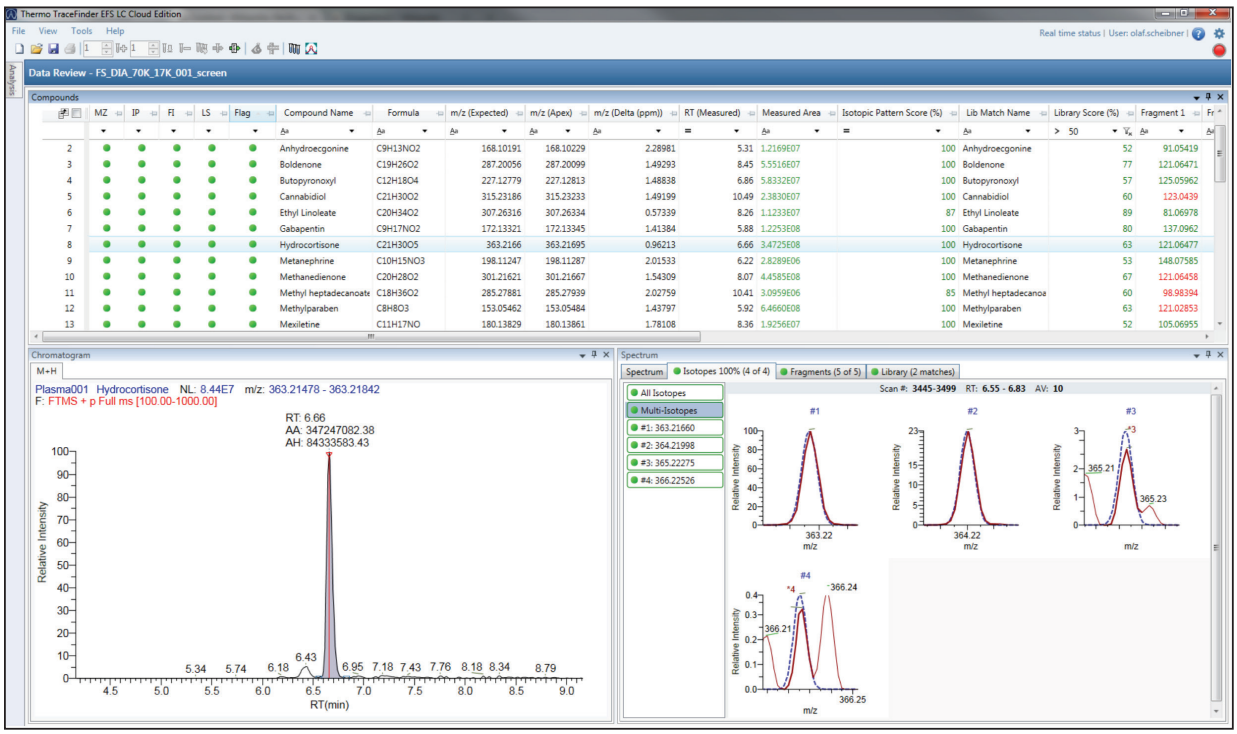

Figure 7. Non-targeted screening result in TraceFinder software.

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