Supporting information

MetExtract II: A software suite for stable isotope assisted untargeted metabolomics metabolomics

Christoph Bueschl[1], Bernhard Kluger[1], Nora KN. Neumann[1], Maria Doppler[1], Valentina Maschietto[2], Gerhard G. Thallinger[3,4], Jacqueline Meng-Reiterer[1,5], Rudolf Krska[1], Rainer Schuhmacher[1]

[1]Center for Analytical Chemistry, Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna, (BOKU), Austria; [2] Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore, Piacenza, Italy; [3]Institute of Computational Biotechnology, Graz University of Technology, 8010 Graz, Austria; [4]Omics Center Graz, BioTechMed Graz, 8010 Graz, Austria; [5]Institute for Biotechnology in Plant Production, Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna, (BOKU), Austria

Corresponding author: Rainer Schuhmacher (rainer.schuhmacher@boku.ac.at)

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1 Biological experiments

In the following, the biological experiments, which have led to the presented datasets, are briefly summarized. For a detailed description please refer to their respective publications. An overview of the five datasets is give in Table S1.

1.1 Dataset AE_Std

The first dataset was taken from the original publication of the MetExtract software for the detection of native and uniformly ^{13}C-labeled substances in LC-HRMS metabolomics data (Supplementary Data available at http://dx.doi.org/10.1093/bioinformatics/bts012)[1]. Briefly summarized, a mix of 15 native and uniformly ^{13}C-labeled fungal substances was prepared in methanol/water (1+1, v+v). The used compounds are listed in Table S2 (#1-15). The mix of the standards was then spiked into a supernatant derived from *Fusarium graminearum* cultivated in liquid minimal medium. It only contained native, non-labeled metabolites and thus served as putatively interfering native contaminants, which should not be detected during automated data processing with AllExtract.

1.2 Dataset AE_Wheat

This dataset exemplifies the suitability of MetExtract II for the global detection of native and U-^{13}C-labeled metabolites in complex biological samples.
Wheat ears of the cultivar "Remus" were grown as hydroponic culture in a medium adapted from Hoagland and Arnon [2]. U-^{13}C labeled wheat of the same cultivar was grown in a tailor made phytolabelbox (ECH Halle) under controlled conditions in $^{13}CO_2$ containing atmosphere (~99% ^{13}C). Samples were taken 96 hours after flowering and immediately frozen in liquid nitrogen. Sample preparation and measurement were carried out as described by Bueschl, et al. [3].

1.3 Dataset TE_DiW

To demonstrate the untargeted screening of tracer-derived secondary metabolites, sample material presented by Kluger, et al. [4] has been re-measured and processed with MetExtract II.
In that study, flowering wheat ears were treated with 100µg of native and uniformly ^{13}C-labeled DON (1+1, v+v). After 108 hours the ears were harvested for further analysis. Details about the performed biological experiment are described by Kluger, et al. [4].

1.4 Dataset ATE_Blanks

These data exemplify the selectivity of the presented MetExtract II data processing strategies. Only the native samples of the datasets AE_Wheat and TE_DiW were analyzed and no ^{13}C-labeled metabolites were present in any of the analyzed samples. Consequently, all metabolites present in those LC-HRMS files can be regarded as putative contaminants for the purpose of testing MetExtract II.
Blank samples were taken from the datasets AE_Wheat and TE_DiW. However, no uniformly ^{13}C-labeled wheat material was added to these blank samples during sample preparation. Additionally, solvent blank samples were also prepared, which neither contained ^{13}C-enriched metabolites nor any native wheat material. These samples were used as blanks for both the AE_Std and TW_DiW datasets.

1.5 Dataset FE_PPAs

The presented LC-HRMS/MS dataset serves to illustrate the last module of the presented software package, FragExtract. For this, LC-MS/MS data of ^{12}C mixture of genotypes:^{13}C-Remus:^{13}C-CM-82036 1:1:1 (v/v/v) of three native and U-^{13}C-labeled phenylpropanoid amides (PPAs) were recorded in a targeted manner.
Native wheat ears of different cultivars (´CM-82036´, Remus, NIL C1, C2, C3 C4) were grown in the green house and inoculated with *Fusarium graminearum* spores at flowering stage and harvested 96h after treatment [5]. U-^{13}C-labeled wheat plants of the cultivar ´CM-82036´ and ´Remus´ plants were grown under controlled conditions in a phytolabelbox (ECH Halle) and infected with *Fusarium graminearum* (10,000 conidia). 96h after Fg inoculation ears were harvested and immediately frozen in liquid nitrogen. Sample preparation was carried out as described by Bueschl, et al. [3]. Native and U-^{13}C extracts were pooled, evaporated under a gentle stream of N_2 gas and re-dissolved resulting in an aliquote of methanol/water/0.1 % formic acid (v/v/v).

Table S1. Overview of the demonstration datasets.

Number	Dataset name	MetExtract II module	Analytes / matrix	Number of data files
1	AE_Std	AllExtract	15 native and U-^{13}C-labeled fungal standards / native fungal culture extract	1
2	AE_Wheat	AllExtract	Extracts of a native and U-^{13}C-labeled wheat ear	5 (+ 7 blanks)
3	TE_WiW	TracExtract	Native and U-^{13}C-labeled DON and its biotransformation products in wheat / Native wheat ear extracts	5 (+ 7 blanks)
4	ATE_Blanks	AllExtract / TracExtract	Matrix blanks: - / Native wheat ear extracts Solvent blanks: - / -	7
5	FE_PPAs	FragExtract	Extracts of a native and U-^{13}C-labeled wheat ear	1

Table S2. List of native and uniformly ^{13}C-labeled fungal metabolites spiked into a native *Fusarium graminearum* extract and summary of detected metabolite ions.

#	Metabolite	Formula	Monoisotopic mass (M)	Δ ppm	RT (min)	C_n	Detected metabolite ions
	X1 [1]				5.38	22	1 unknown [*m/z* 445.1597]
1	Tetracycline	$C_{22}H_{24}N_2O_8$	444.1533	-1.83	6.36	22	$[M+H]^+$, $[M+Na]^+$
2	Deoxynivalenol (DON)	$C_{15}H_{20}O_6$	296.1260	-1.67	6.61	15	$[M+H]^+$ [2]
3	3-Acetyldeoxynivalenol	$C_{17}H_{22}O_7$	338.1366	-2.21	9.17	17	$[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$
	X2 [1]		389.1845 [3]		9.62	22	$[M+H]^+$, $[M+Na]^+$
4	Aflatoxin G1	$C_{17}H_{12}O_7$	328.0583	-2.32	10.26	17	$[M+H]^+$, $[M+Na]^+$, $[2M+Na]^+$, 4 further ion species
5	Fumonisin B1	$C_{34}H_{59}NO_{15}$	721.3885	-1.13	10.72	34	$[M+H]^+$, $[M+Na]^+$, 1 further ion species
	X3 [1]				10.83	17	1 not assignable ion species [*m/z* 701.2193]
6	Aflatoxin B1	$C_{17}H_{12}O_6$	312.0634	-1.76	10.93	17	$[M+H]^+$, $[M+Na]^+$, $[2M+Na]^+$, 4 further ion species
7	Fumonisin B3	$C_{34}H_{59}NO_{14}$	705.3936	-0.94	11.32	34	$[M+H]^+$, 1 further ion species
8	Fumonisin B2	$C_{34}H_{59}NO_{14}$	705.3936	-1.48	11.74	34	$[M+H]^+$, 1 further ion species
9	HT-2 toxin	$C_{22}H_{32}O_8$	424.2097	-1.71	11.93	22	$[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$, 1 further ion species
10	Griseofulvin	$C_{17}H_{17}O_6Cl$	352.0713	-1.47	11.93	17	$[M+H]^+$ [2], $[M+Na]^+$ [2], $[2M+H]^+$ [2], $[2M+Na]^+$ [2], 1 further ion species [2], 1 further ion species
11	T2 toxin	$C_{24}H_{34}O_9$	466.2203	-1.83	12.55	24	$[M+NH_4]^+$, $[M+Na]^+$, $[M+K]^+$, 5 further ion species
12	Roquefortine C	$C_{22}H_{23}N_5O_2$	389.1852	-2.18	13.12	22	$[M+H]^+$, $[M+Na]^+$
13	Zearalenone	$C_{18}H_{22}O_5$	318.1467	-1.74	13.44	18	$[M-H_2O+H]^+$, $[M+H]^+$, $[M-H_2O+Na]^+$, $[M+Na]^+$, 2 further ion species
14	Sterigmatoxystin	$C_{18}H_{12}O_6$	324.0634	-1.67	13.69	18	$[M+H]^+$, $[M+Na]^+$, 1 further ion species

15	Ochratoxin A	$C_{20}H_{18}NO_6Cl$	403.0823	-1.78	13.80	20	$[M+H]^{+}$ [2], $[M+Na]^{+}$ [2], $[M+2Na-H]^{+}$, 2 further ion species

1… This detected ion could not be referenced to one of the spiked fungal substances
2… This ion was detected or annotated as a ^{37}Cl isotopolog
3… Experimentally determined mass, not theoretically calculated

2 LC-HRMS analysis

2.1 Dataset AE_Std

The mixed sample consisting of the 15 native and uniformly ^{13}C-labeled fungal substances and the native *Fusarium graminearum* background was measured using an LTQ Orbitrap XL instrument (Thermo Fisher Scientific). LC-HRMS and HPLC parameters were identical to those described by Lehner, et al. [6]. MS spectra were recorded in full scan mode with a resolving power setting of 60,000 (FHWM @ *m/z* 400) in the positive ionization mode. Further details about the analytical method are described by Bueschl, et al. [1].

2.2 Datasets AE_Wheat, TE_DiW, ATE_Blanks

The samples were measured on an Orbitrap Exactive Plus instrument (Thermo Fisher Scientific). The electrospray ionization source was operated in fast-polarity switching mode. The polarity was switched after each MS scan. Further details about the analytical measurements are described by Kluger, et al. [7].
For demonstration of the algorithm, the samples of Kluger, et al. [4] have been reused and measured once more. Samples were more than 4 years old so it was expected that some metabolites had degraded and could not be detected any longer.

2.3 Dataset FE_PPAs

The pooled, U-^{13}C-standardized wheat sample was analyzed on an LTQ Orbitrap XL instrument (Thermo Fisher Scientific) in a targeted approach. For each of the three PPAs two successive LC-MS/MS scan events were defined using a) the monoisotopic native (M) and b) the fully ^{13}C-labeled form (M') as precursor ions. All MS/MS precursor ions are listed in Table S3.

Table S3. List of defined MS/MS precursors in the dataset FE_PPAs

#	Metabolite	Abbreviation	*m/z*(M)	*m/z*(M')	C_n(M)	Rt for MS/MS scan events	Sum formula
1	Coumaroyl-putrescine	CouPut	235.1441	248.1877	13	7.3-8.0	$C_{13}H_{18}O_2N_2$
2	Coumaroyl-agmatine	CouAgm	277.1659	291.2128	14	9.8-10.4	$C_{14}H_{20}O_2N_4$
3	Coumaroyl-serotonine	CouSer	323.1390	342.2027	19	16.0-16.5	$C_{19}H_{18}O_3N_2$

3 Data processing

Raw LC-HRMS data were converted to the mzXML format using the msConvert tool of the ProteoWizard toolbox (version 3.0.8789)[8]. In case of the dataset AE_Std the already converted mzXML file was used (conversion performed with ReAdW (version 4.0.2 (build July 1 2008 14:23:37))). For FragExtract, the raw data were converted to the mzML format since the respective precursor *m/z* values for LC-HRMS/MS scans are preserved after data conversion, which is not the case if raw data files are converted into the mzXML format. An overview of the used data processing parameters is given in Table S4. The raw-data can be downloaded from https://metabolomics-ifa.boku.ac.at/metextractII/.

3.1 Dataset AE_Std

Wherever possible, data processing parameters were kept identical to the previous version of MetExtract.[1]

3.2 Dataset AE_Wheat

Data processing of the samples derived from native and U-^{13}C-labeled wheat ears were processed with the AllExtract module. Both the M+1 and M'-1 isotopologs of each metabolite as well as the accuracy of their relative abundances in respect to the number of carbon atoms per ion were checked. Only feature pairs passing these criteria were used for further analysis.

3.3 Dataset TE_DiW

Data files from samples investigating the biotransformation of deoxynivalenol in wheat were processed with the TracExtract module. Parameter settings were kept similar to those of the dataset AE_Wheat. The number of carbon atoms to search for was limited to such numbers, which can be expected for the used $^{13}C_{15}$-labeled tracer. Moreover, 30 carbon atoms were also included to search for feature pairs consisting of dimers with two $^{13}C_{15}$-labeled deoxynivalenol tracer molecules per putative metabolite ion.

3.4 Dataset ATE_Blanks

Data processing of the matrix and solvent blank samples was performed equivalent to the datasets AE_Wheat and ET_DiW. After data processing, the blank and solvent blank samples were separately inspected for false-positives.

3.5 Dataset FE_PPAs

LC-HRMS/MS data processing of the three target ion pairs (M and M') was performed with FragExtract according to the settings specified in Table S4.

Table S4. Overview of the used data processing parameter settings and generated results. Empty fields indicate parameters that were not applicable for the processing of the respective dataset.

Step	Parameter	AE_Std	AE_Wheat	TE_DiW	ATE_Blanks		FE_PPAs
	Instrument	LTQ Orbitrap XL	Orbitrap Exactive plus	Orbitrap Exactive plus	Orbitrap Exactive plus		LTQ Orbitrap XL
	Ion mode(s)	Pos.	Pos. / Neg.	Pos. / Neg.	Pos. / Neg.		Pos.
Single file processing	Module	AllExtract	AllExtract	TracExtract	AllExtract	TracExtract	FragExtract
	Isotopic enrichment % (N / L)	98.9 / 99.5	98.9 / 98.6	98.9 / 99.5	Samples were processed with the same settings as the datasets AE_Wheat and TE_DiW		98.9 / 98.6
	Δ *m/z*	1.00335 [1]	1.00335	1.00335			1.00335
	Min / max C_n atoms	3-70 [1]	5-60	10-15, 30 [5]			
	Scan range (minutes)	2-37 [1]	3-30	3-30			
	Intensity threshold (≥)	1,000 [1]	100,000	100,000			1,000
	Intensity cutoff (≤)	0	10,000	10,000			
	Consider isotopolog abundance	No	No	No			No
	Mass deviation (± ppm)	4 [1]	3	3			50
	Isotopologs verified (N / L)	2 / 2 [1]	2 / 2	2 / 2			1 / 1
	Max. isotopolog ratio error % (N / L)	25 / 25 [1]	10 / 10	35 / 10			50
	Clustering (ppm)	4 [1]	10	10			
	Min. number of spectra	3	4	4			
	EIC width (± ppm)	4 [1]	8	8			5
	Peak width (min / max scans)	3 / 19	13 / 31	13 / 31			
	Peak matching (scans)	10 ◦	10	10			
	Min. peak correlation	0.75 ◦	0.75	0.75			
	Min. convolution correlation	0.8	0.85	0.85			
Bracketing	Max. *m/z* width (± ppm)		6	6			
	Max. time window (min)		0.15	0.15			
	Min. connections for convolution		4	2			
	Integration max. time difference (min)		0.15	0.15			
Results	Paired signals	1,026	60,629 [3]	1,105 [3]	1,581 [3]	203 [3]	7 [3]
	Detected feature pairs	72	2,430 [4]	84 [4]	2 [3]	0 [3]	7 [3]
	Convoluted feature groups	17	506 [4]	21 [4]	2 [3]	0 [3]	7 [3]

N… Native metabolite ion
L… U-^{13}C-labeled metabolite ion
1… Setting was kept identical to Bueschl, et al. [1]
2… Setting could not be kept identical to Bueschl, et al. [1]
3… On average in all replicates
4… Bracketed results
5… 30 carbon atoms were used to also detect dimer ions containing two ^{13}C-labeled tracer entities (e.g. $[2M+H]^+$)

4 Generated results

4.1 Dataset AE_Std

All 15 fungal standards were successfully detected with the AllExtract module. An overlay of the EICs of all found feature pairs is depicted in Figure S3. The chloride-37 isotopes (^{37}Cl) of the metabolites griseofulvin and ochratoxin A were successfully annotated as such isotopologs and convoluted to their respective feature groups. Two substances (HT-2 toxin and griseofulvin) were incorrectly convoluted into one feature group since these two metabolites show an almost complete co-elution in the LC-HRMS analysis. In addition to the 15 spiked fungal metabolites, 4 feature pairs corresponding to 3 unknown metabolites (X1-X3) were detected, which clearly showed the expected native and U-^{13}C-labeling-derived isotope patterns. These metabolites most probably represent contaminants / impurities of one or more fungal standard compounds. X1 and X2 had also been reported with the previous MetExtract version, while X3 was only detected with the presented MetExtract II.

4.2 Dataset AE_Wheat

The AllExtract module detected 506 metabolites in the dataset AE_Wheat originating from a total of 2,430 feature pairs in both the positive and / or the negative ionization modes. Each such ion was verified using the unique isotope patterns of the native and the uniformly ^{13}C-labeled metabolite forms as well as their very similar chromatographic peak shapes and retention times. Figure S4 exemplifies a single metabolite for which in total 35 ions were detected.
Of the 2,430 feature pairs, 922 were detected in the negative and 1,508 were detected in the positive ionization mode (Figure S5A). Among those, 644 feature pairs were annotated with predefined ion species, commonly observed in LC-HRMS. The ion species $[M+H]^+$ (185 times) and $[M-H]^-$ (184 times) were the most commonly annotated ion species followed by $[M+Na]^+$ (109 times) and $[M+NH_4]^+$ (74 times). Only 17 feature pairs were detected that carried two charges per ion. The 2,430 feature pairs were grouped to a total of 506 metabolites. Of these, 208 (41%) had feature pairs in both the positive and the negative ionization modes. Furthermore, 105 (21%) and 193 (38%) feature groups were only annotated with ions of the negative or positive ionization modes respectively. While 377 detected feature groups consisted of less than 6 feature pairs, 129 feature groups consisted of 6 or more feature pairs.

4.3 Dataset TE_DiW

With the applied parameter settings, a total of 21 biotransformation products (84 feature pairs) of deoxynivalenol-derived biotransformation products in wheat were detected by the TracExtract module (Figure S6). All detected biotransformation products still had 15 carbon atoms from the investigated DON-tracer, indicating an intact carbon skeleton for all detected DON derivatives.
However, since the samples were taken from the former experiment of Kluger, et al. [4], metabolite degradation was expected. Consequently, the presented data was not suited for biological interpretation.

4.4 Dataset ATE_Blanks

Native wheat and solvent blank samples processed with AllExtract in the dataset AE_Wheat
In the blank samples on average only 2 incorrectly detected feature pairs were found when these samples were processed with the same settings as the dataset AE_Wheat. This low number of false-positives demonstrates the high selectivity of AllExtract and can be attributed to the strict filtering of the carbon-isotope pattern verification as well as the chromatographic peak correlation verification. Moreover, in the re-integration step subsequently to feature pair bracketing, MetExtract II also searches for all detected ions in a targeted manner. In the final data matrix only peak areas for the ions of the native metabolite forms were detected but almost no peaks were re-integrated for the respective uniformly ^{13}C-labeled metabolite ions. In the solvent blank samples false-positive chromatographic peaks were integrated only occasionally for the native or the uniformly ^{13}C-labeled metabolite ions.

Native wheat samples processed with TracExtract in the dataset TE_DiW
With the dataset TE_DiW the original aim was to detect as many biotransformation products of deoxynivalenol in wheat plants as possible. No false-positive feature pairs were detected in these samples when processed with the same settings as the dataset TE_DiW. This reduction can be explained by the number of carbon atoms TracExtract was instructed to search for. While in the dataset AE_Wheat the respective parameter was set to 3-60, in the TE_DiW dataset only 10-15 and 30 carbon atoms were searched for.

4.5 Dataset FE_PPAs

In total, 20 fragment ion pairs were annotated by the FragExtract module for the three metabolites. Each of these fragments was successfully annotated with a unique sum formula as well as the loss relative to its precursor ion. A manual inspection of the results using the chromatographic peak shapes showed they were similar confirming that all paired fragment ions of the native and the U-^{13}C labeled precursor ions of the same metabolites are true fragments of the investigated metabolites. The results are shown exemplarily for p-coumaroyl-agmatine in Table S5 and are further illustrated in Figure S7.

Table S5. Overview of the detected fragment ions for the precursor p-coumaroyl-agmatine in the dataset FE_PPAs.

#	*mz*(F)	Relative Intensity (%)	C_n	Adduct	Sum Formula	Neutral loss to parent ion
0	114.1026	9.5	5	$[M]^+$	$C_5H_{12}N_3$	$C_9H_9NO_2$
1	115.0866	5.6	5	$[M]^+$	$C_5H_{11}ON_2$	$C_9H_{10}N_2O$
2	147.0441	6.0	9	$[M]^+$	$C_9H_7O_2$	$C_5H_{14}N_4$
3	217.134	24.5	13	$[M]^+$	$C_{13}H_{17}ON_2$	CH_4N_2O
4	218.1178	4.6	13	$[M]^+$	$C_{13}H_{16}O_2N$	CH_5N_3
5	235.1445	1.9	13	$[M]^+$	$C_{13}H_{19}O_2N_2$	CH_2N_2
6	260.1400	12.4	14	$[M]^+$	$C_{14}H_{18}O_2N_3$	H_3N
7	**277.1665 (Precursor)**	**100**	**14**	**$[M]^+$**	**$C_{14}H_{21}O_2N_4$**	-

References

(1) Bueschl, C.; Kluger, B.; Berthiller, F.; Lirk, G.; Winkler, S.; Krska, R.; Schuhmacher, R. *Bioinformatics* **2012**, *28*, 736-738.
(2) Hoagland, D. R.; Arnon, D. i. *Water culture method for growing plants without soil*: Berkeley, Calif.: University of California, College of Agriculture, Agricultural Experiment Station, 1938.
(3) Bueschl, C.; Kluger, B.; Lemmens, M.; Adam, G.; Wiesenberger, G.; Maschietto, V.; Marocco, A.; Strauss, J.; Bodi, S.; Thallinger, G. G.; Krska, R.; Schuhmacher, R. *Metabolomics* **2014**, *10*, 754-769.
(4) Kluger, B.; Bueschl, C.; Lemmens, M.; Berthiller, F.; Haubl, G.; Jaunecker, G.; Adam, G.; Krska, R.; Schuhmacher, R. *Anal. Bioanal. Chem.* **2013**, *405*, 5031-5036.
(5) Warth, B.; Parich, A.; Bueschl, C.; Schoefbeck, D.; Neumann, N. K.; Kluger, B.; Schuster, K.; Krska, R.; Adam, G.; Lemmens, M.; Schuhmacher, R. *Metabolomics* **2015**, *11*, 722-738.
(6) Lehner, S. M.; Neumann, N. K.; Sulyok, M.; Lemmens, M.; Krska, R.; Schuhmacher, R. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* **2011**, *28*, 1457-1468.
(7) Kluger, B.; Bueschl, C.; Neumann, N.; Stuckler, R.; Doppler, M.; Chassy, A. W.; Waterhouse, A. L.; Rechthaler, J.; Kampleitner, N.; Thallinger, G. G.; Adam, G.; Krska, R.; Schuhmacher, R. *Anal. Chem.* **2014**, *86*, 11533-11537.
(8) Kessner, D.; Chambers, M.; Burke, R.; Agusand, D.; Mallick, P. *Bioinformatics* **2008**, *24*, 2534-2536.

Supplementary figures

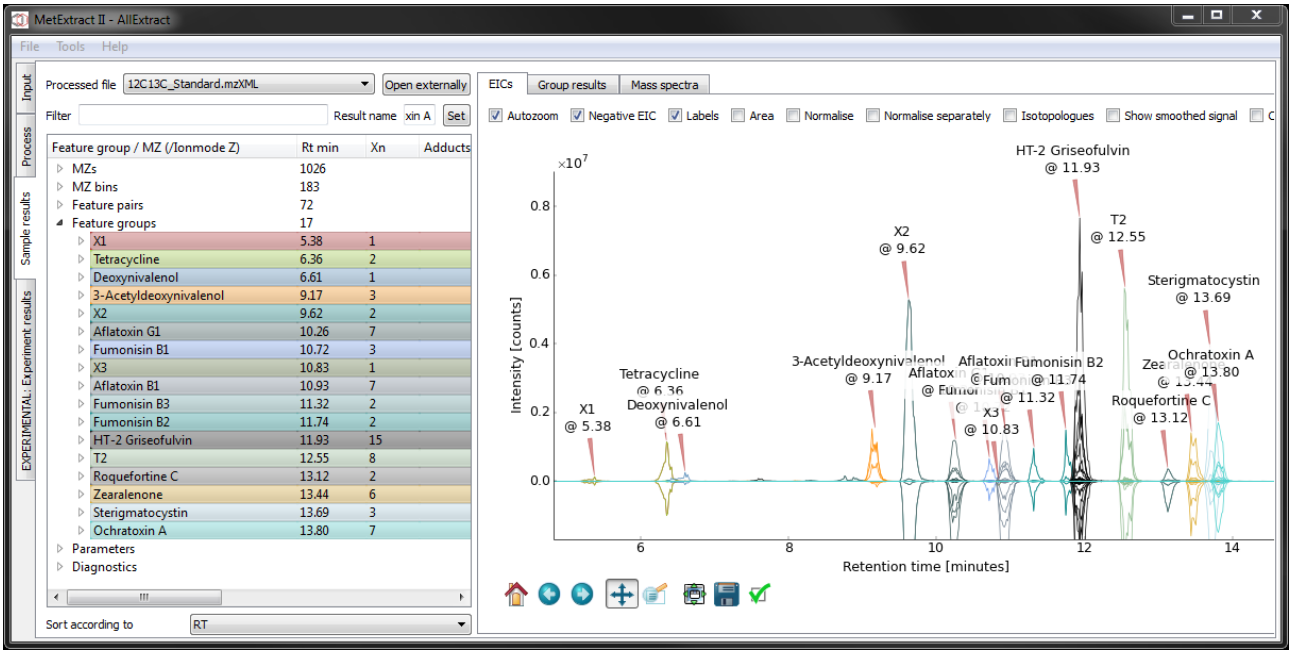


Figure S1. Screenshot of the MetExtract II software's results panel showing the detected metabolites for the dataset AE_Std. The EICs of all detected ions of the 17 metabolites are overlaid in the plot on the right side. Native, monoisotopic EIC traces are illustrated with positive intensities while the corresponding ion traces of the fully ^{13}C-labeled analogues are depicted with negative intensities. Metabolite names were set manually after the data processing.

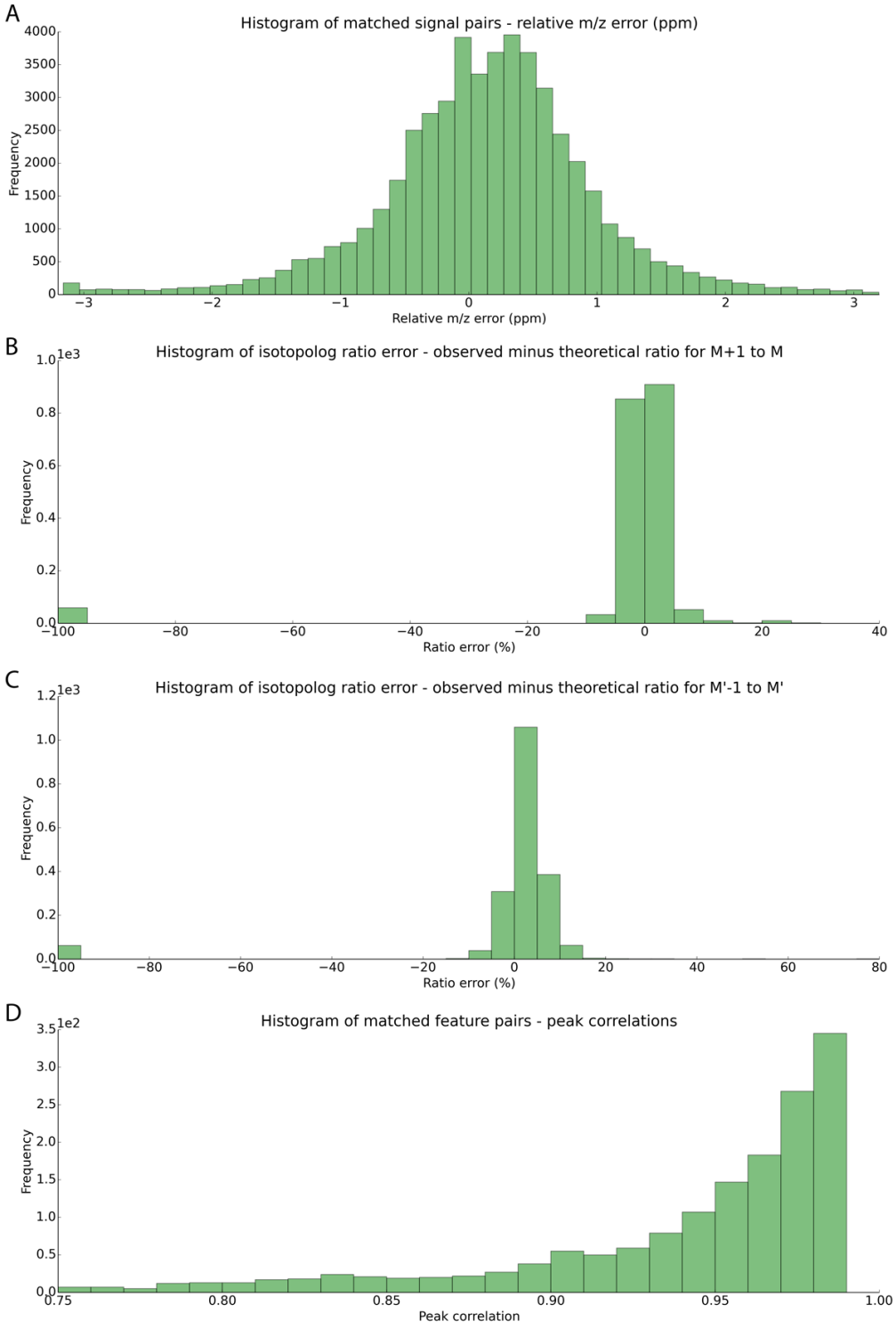


Figure S2. Diagnostic plots generated for a sample containing native and U-^{13}C-labeled wheat from the dataset AE_Wheat. A shows the intra-scan mass difference deviation between signals of M and M' ions. B illustrates the accuracy of ratios of M+1 to M signals for all detected feature pairs, when the assigned number of C-atoms per detected ion is used as a reference. C shows a similar plot for the isotopolog ratios of the signals M'-1 to M'. The bar at -100% in B and C depict the isotopolog ratios for scans in which either M+1 or M'-1 could not be detected (i.e. chromatographic peak borders). D depicts the distribution of peak correlations for the two chromatographic peaks of M and M' for each putatively detected feature pair. These diagnostic plots confirm, that the used settings are valid and that some parameters could even be further improved marginally (e.g. the correlation cutoff could be increased to 0.85 to remove potentially false-positives).

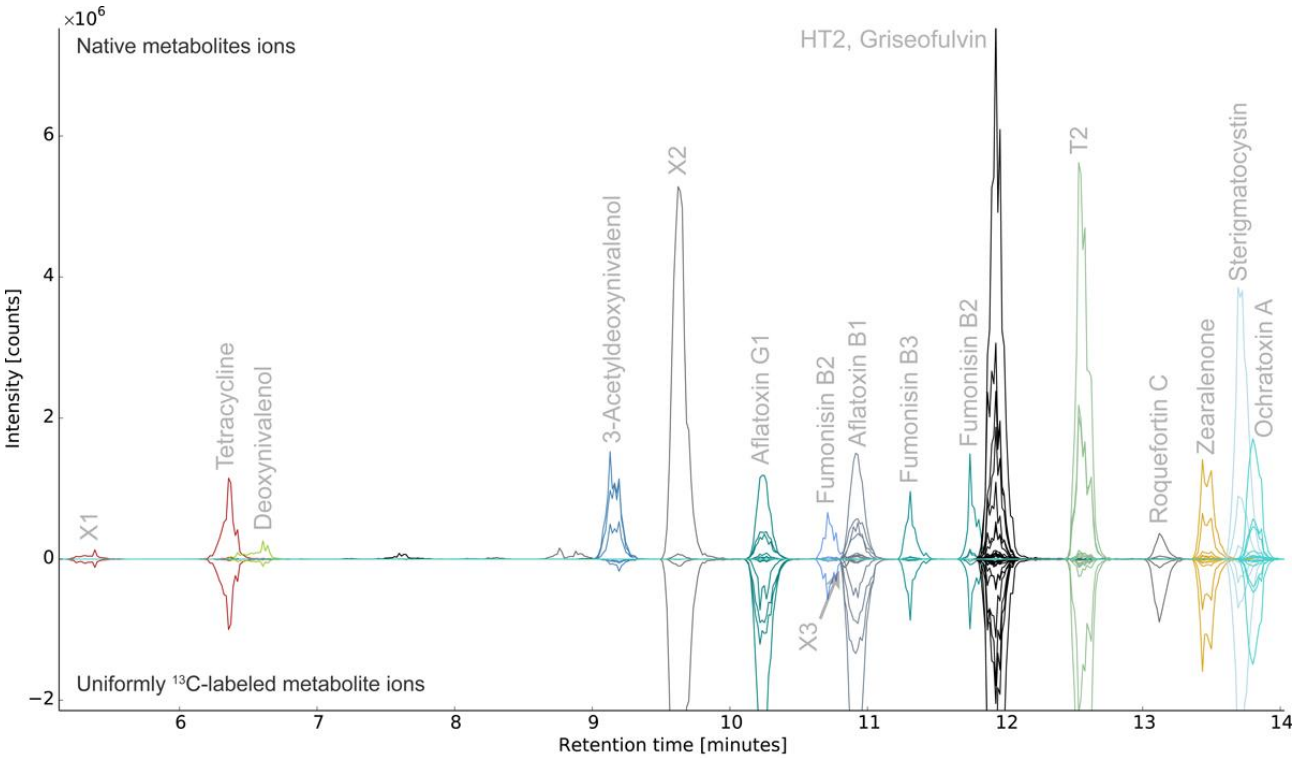


Figure S3. Illustration of the detected fungal metabolite standards in the dataset AE_Std

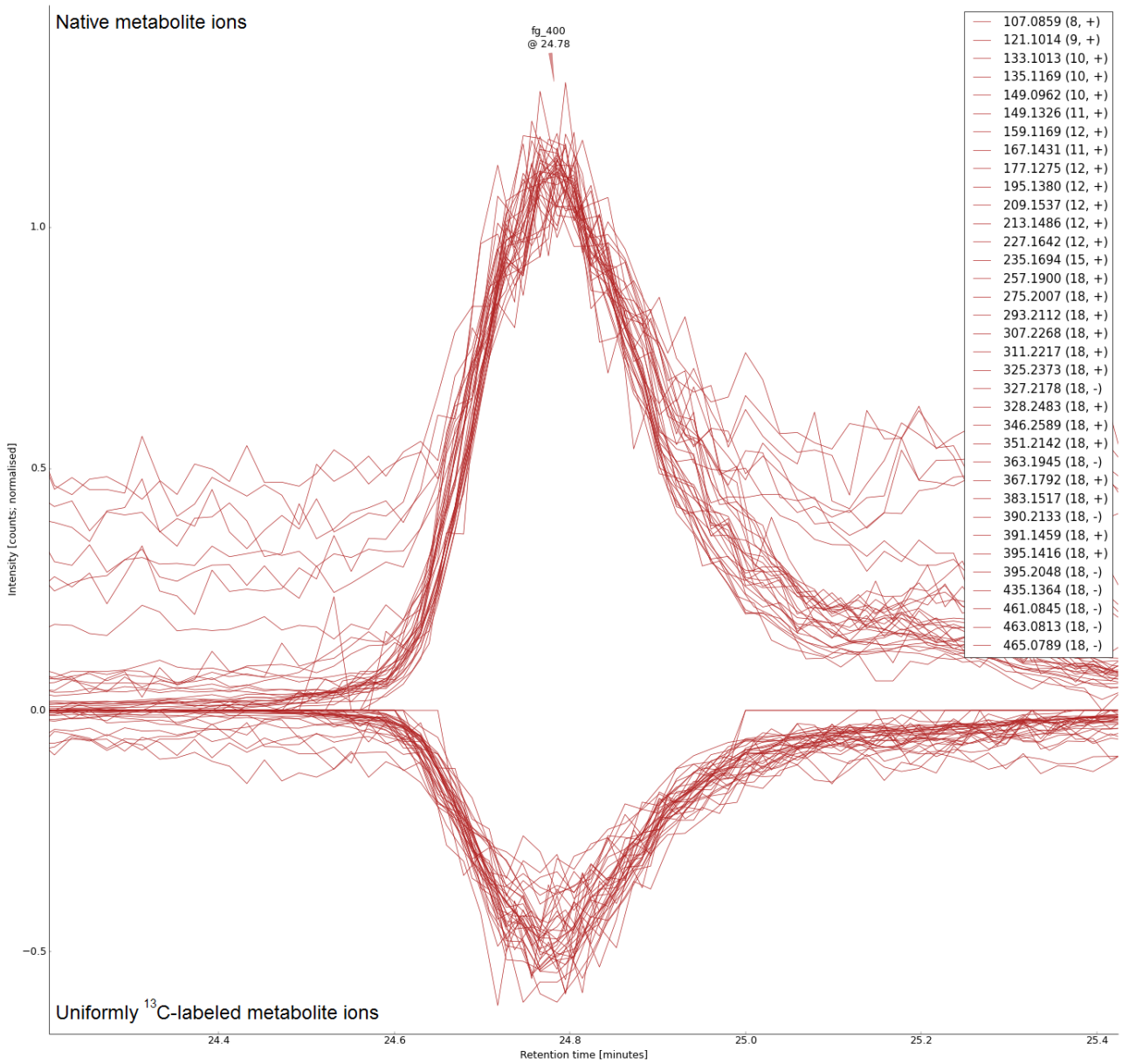


Figure S4. Illustration of a wheat metabolite detected as 35 different ion species. The principal ion with the *m/z* value 327.21787 has been annotated with 18 carbon atoms, an abundance of 1.1*E8 counts and has been assigned the $[M-H]^-$ ion species. Peak heights have been normalized (average intensity at the peaks' centers has been set to 1 and the EIC traces of each feature pair have been scaled accordingly) in order to improve the illustration of these co-eluting feature pairs.

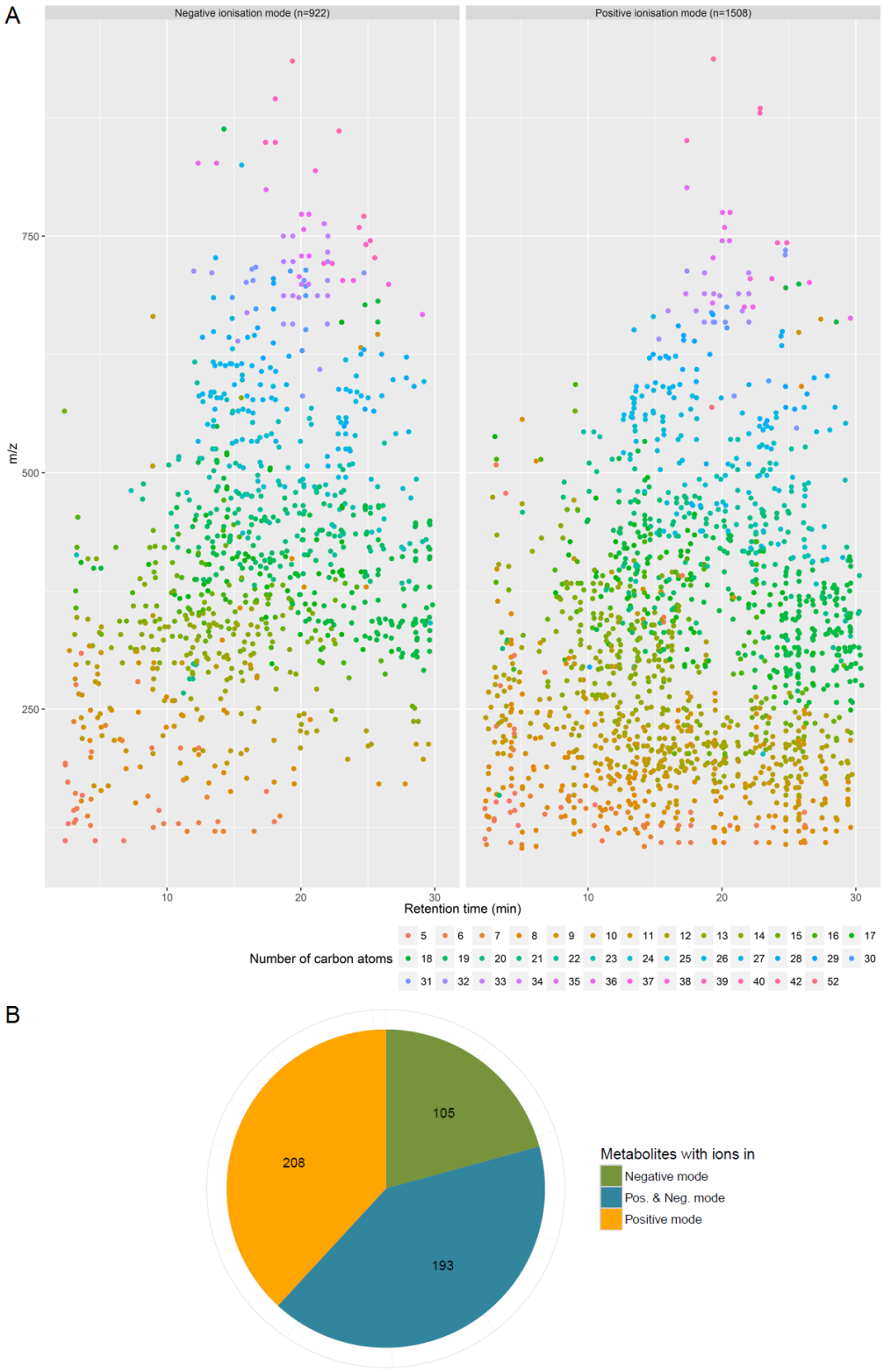


Figure S5. A shows the detected feature pairs in the dataset AE_Wheat. Each dot represents one feature pair and its color corresponds to the number of carbon atoms calculated from the Δ*m/z* value between the uniformly ^{13}C-labeled and the monoisotopic native isotopolog of the respective metabolite ion. B shows summary of the detected metabolites' ionization modes. Of the 506 metabolites, 62% were detected as negative mode ions and 79% of the metabolites showed ions in the positive mode. 193 metabolites (38%) showed were detected as ions in both ionization modes.

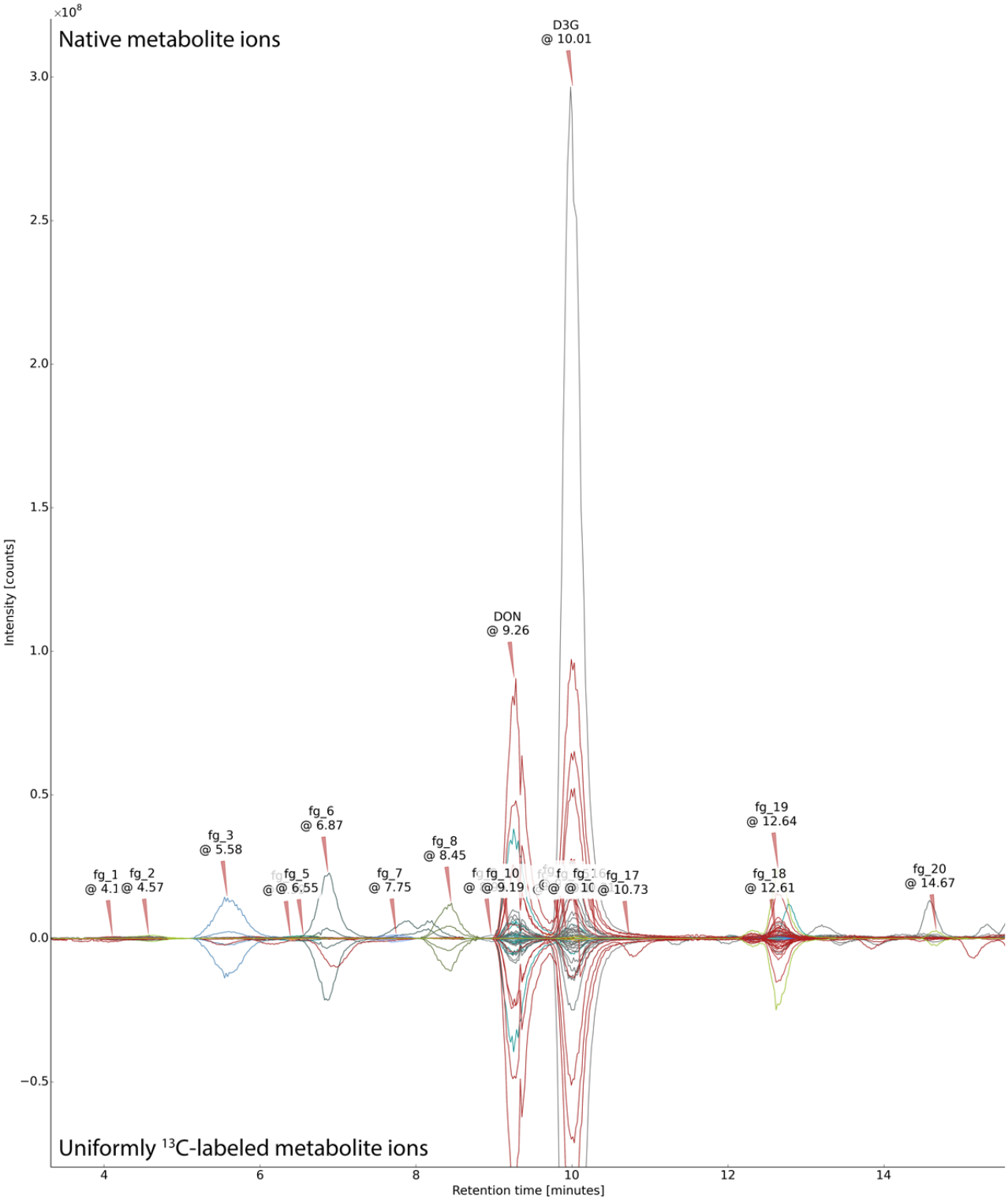
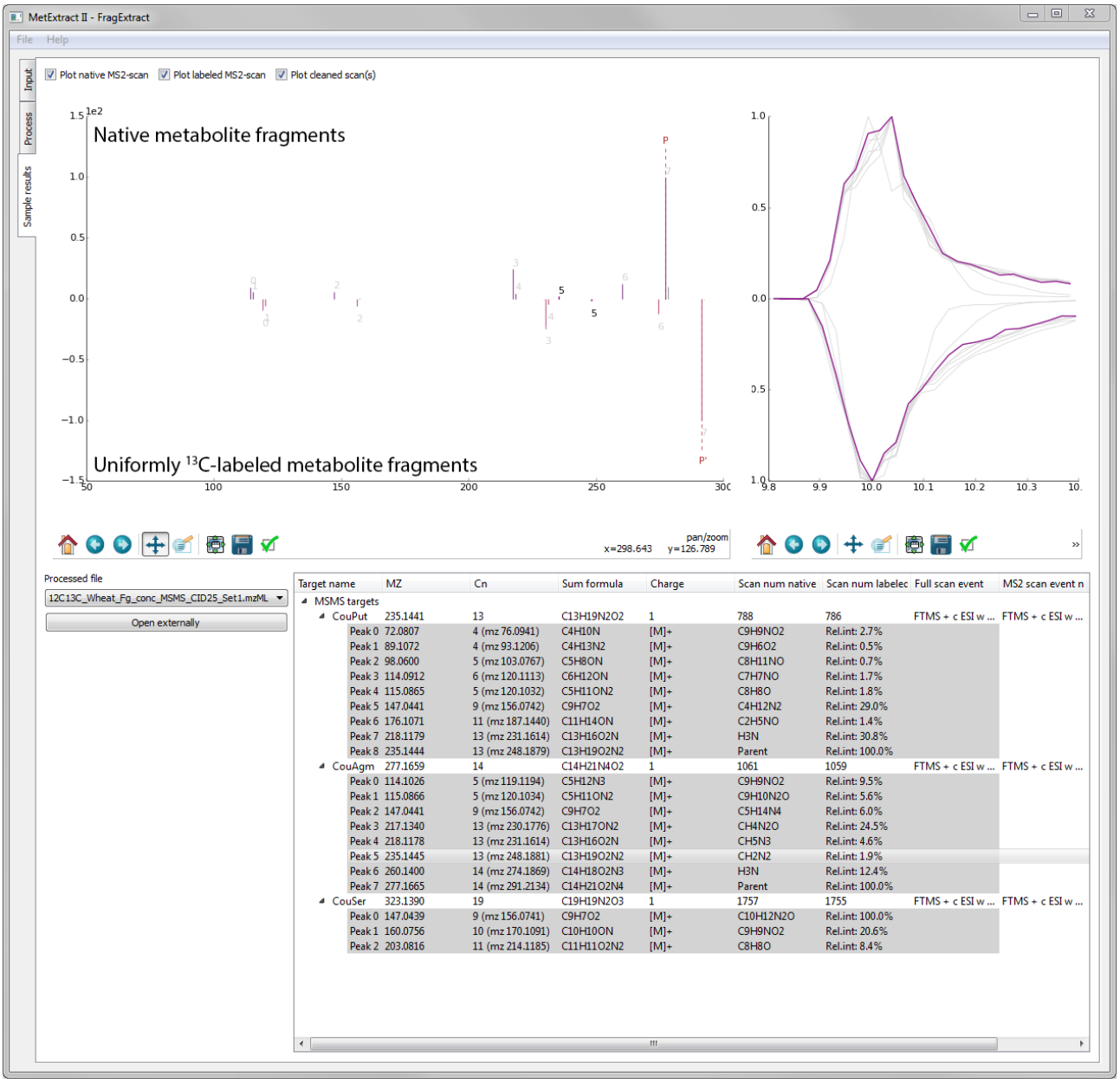


Figure S6. Illustration of detected biotransformation products in the dataset TE_DiW shown as an overlay of their EICs.



Target name	MZ	Cn	Sum formula	Charge	Scan num native	Scan num labelec	Full scan event	MS2 scan event n
MSMS targets								
CouPut	235.1441	13	C13H19N2O2	1	788	786	FTMS + c ESI w ...	FTMS + c ESI w ...
Peak 0	72.0807	4 (mz 76.0941)	C4H10N	[M]+	C9H9NO2	Rel.int: 2.7%		
Peak 1	89.1072	4 (mz 93.1206)	C4H13N2	[M]+	C9H6O2	Rel.int: 0.5%		
Peak 2	98.0600	5 (mz 103.0767)	C5H8ON	[M]+	C8H11NO	Rel.int: 0.7%		
Peak 3	114.0912	6 (mz 120.1113)	C6H12ON	[M]+	C7H7NO	Rel.int: 1.7%		
Peak 4	115.0865	5 (mz 120.1032)	C5H11ON2	[M]+	C8H8O	Rel.int: 1.8%		
Peak 5	147.0441	9 (mz 156.0742)	C9H7O2	[M]+	C4H12N2	Rel.int: 29.0%		
Peak 6	176.1071	11 (mz 187.1440)	C11H14ON	[M]+	C2H5NO	Rel.int: 1.4%		
Peak 7	218.1179	13 (mz 231.1614)	C13H16O2N	[M]+	H3N	Rel.int: 30.8%		
Peak 8	235.1444	13 (mz 248.1879)	C13H19O2N2	[M]+	Parent	Rel.int: 100.0%		
CouAgm	277.1659	14	C14H21N4O2	1	1061	1059	FTMS + c ESI w ...	FTMS + c ESI w ...
Peak 0	114.1026	5 (mz 119.1194)	C5H12N3	[M]+	C9H9NO2	Rel.int: 9.5%		
Peak 1	115.0866	5 (mz 120.1034)	C5H11ON2	[M]+	C9H10N2O	Rel.int: 5.6%		
Peak 2	147.0441	9 (mz 156.0742)	C9H7O2	[M]+	C5H14N4	Rel.int: 6.0%		
Peak 3	217.1340	13 (mz 230.1776)	C13H17ON2	[M]+	CH4N2O	Rel.int: 24.5%		
Peak 4	218.1178	13 (mz 231.1614)	C13H16O2N	[M]+	CH5N3	Rel.int: 4.6%		
Peak 5	235.1445	13 (mz 248.1881)	C13H19O2N2	[M]+	CH2N2	Rel.int: 1.9%		
Peak 6	260.1400	14 (mz 274.1869)	C14H18O2N3	[M]+	H3N	Rel.int: 12.4%		
Peak 7	277.1665	14 (mz 291.2134)	C14H21O2N4	[M]+	Parent	Rel.int: 100.0%		
CouSer	323.1390	19	C19H19N2O3	1	1757	1755	FTMS + c ESI w ...	FTMS + c ESI w ...
Peak 0	147.0439	9 (mz 156.0741)	C9H7O2	[M]+	C10H12N2O	Rel.int: 100.0%		
Peak 1	160.0756	10 (mz 170.1091)	C10H10ON	[M]+	C9H9NO2	Rel.int: 20.6%		
Peak 2	203.0816	11 (mz 214.1185)	C11H11O2N2	[M]+	C8H8O	Rel.int: 8.4%		

Figure S7. Screenshot of the FragExtract module with the processing results of the metabolite CouAgm for which 8 fragment peaks between MS/MS spectra of the native and uniformly ^{13}C-labeled ion forms have been detected. The peak with the number 5 (*m/z* 235.1445, relative intensity of 1.9%) was successfully matched between the two MS/MS spectra and is highlighted. It was annotated with the sum formula $C_{13}H_{19}O_2N_2$. The top-right chromatographic peak plot shows that all detected fragment signals have similar chromatographic peaks.