CPEXtract, a Software for the Automated Tracer-Based Pathway Specific Screening of Secondary Metabolites in LC-HRMS Data

Bernhard Seidl, Rainer Schuhmacher*, Christoph Bueschl

Institute for Bioanalytics and Agro-Metabolomics, Department of Agrobiotechnology (IFAPA-Tulln), University of Natural Resources and Life Sciences, Vienna, Konrad Lorenz Str. 20, A-3430 Tulln, Austria

ABSTRACT: The use of stable isotopically labeled tracers is a long-proven way of specifically detecting and tracking derived metabolites through a metabolic network of interest. While recently developed stable isotope assisted methods and associated, supporting data analysis tools have greatly improved untargeted metabolomics approaches, no software tool is currently available that allows to automatically search LC-HRMS chromatograms for completely free user-definable isotopolog patterns expected for the metabolism of labeled tracer substances. Here we present Custom Pattern Extract (CPEXtract), a versatile software tool that allows for the first time the high-throughput search for user-defined isotopolog patterns in LC-HRMS data. The patterns can be specified via a set of rules including the presence or absence of certain isotopologs, their relative intensity ratios as well as chromatographic co-elution. Each isotopolog pattern satisfying the respective rules is verified on a MS-scan level and also in the chromatographic domain. The CPEXtract algorithm allows the use of both labeled tracer compounds in non-labeled biological samples as well as a reversed tracer approach, employing non-labeled tracer compounds along with globally labeled biological samples.

In a proof of concept study we searched for metabolites specifically arising from the malonate pathway of the filamentous fungi Fusarium graminearum and Trichoderma reesei. 1,2,3-13C3-malonate acid diethyl ester and native malonic acid monomethyl ester were used as tracers. We were able to reliably detect expected fatty acids and known polyketides. In addition, up to 189 and 270 further, unknown metabolites presumably including novel polyketides were detected in the F. graminearum and T. reesei culture samples respectively, all of which exhibited the user-predicted isotopolog patterns originating from the malonate tracer incorporation. The software can be used for every conceivable tracer approach. Furthermore, the rule sets can be easily adapted or extended if necessary. CPEXtract is available free of charge for non-commercial use at https://metabolomics-ifas.boku.ac.at/CPEXtract.

INTRODUCTION

Untargeted metabolomics aims at the unbiased detection of all metabolites produced by a biological system that are covered by the respective analytical method used. Typically both qualitative and quantitative differences in metabolites resulting from natural fluctuations, genetic modifications (e.g. WT versus knock-out) or experimental biotic or abiotic stress factors (e.g. untreated versus treated in any form) are then examined [1]. Nowadays, besides nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) is the most commonly used analytical technique to cope with this task. LC-HRMS allows the detection of a large number of different metabolites in the samples. However, usually only few of these can be identified by comparison with reference standards [2]. Moreover, it is often not even possible to reliably differentiate between signals deriving from contaminants or artefacts and those of truly biological metabolites [3]. To encounter this problem, stable isotopically labeled samples, biological material or tracer molecules can be employed. The ingenious basic principle here is that labeling is incognito for the organism, i.e. labeled substances are metabolized almost the same way as unlabeled substances in biochemical reactions, but the label can be easily traced in analytical measurements [4, 5]. Stable isotope-assisted (SIA) workflows were used as early as the 1950s in comprehensive studies of metabolic pathways, where the progression of biochemical signaling pathways and metabolic intermediates was investigated by tracking changes in the isotopic composition of metabolites of the metabolic pathway under study over time. This made it possible to study metabolic pathways and networks or to investigate the metabolic fate of substances (e.g. drugs or toxins) into intermediate or end products. Typically, 13C, 15N or 2H isotopes are used for tracking derived metabolites through a given metabolic network of interest [5-9]. Additionally, the labeling information can also be used for improved unknown compound annotation, e.g. by means of interpretation of SIA tandem mass spectrometry data [10].

Meanwhile software-supported approaches for the untargeted search for tracer-derived compounds also are commonly used in metabolomics, where in contrast to the more specific biosynthetic pathway studies, a snapshot of the entire metabolic state that can be captured by the method used is analyzed [1]. In the latter case, the artificial isotope patterns caused by the metabolism of the labeled tracer are used to reliably track all tracer-derived metabolites that contain the entire tracer or parts thereof.

However, especially in untargeted SIA metabolomics studies, the huge amounts of raw LC-HRMS data do not allow the comprehensive, manual evaluation anymore. Therefore, a variety of (semi)automated SIA methods and data evaluation tools have recently been developed and greatly helped to improve untargeted metabolomics approaches. Examples of such tools are MetExtract II [11], X13CMS [12], HiTIME [13], geoRge [14], ALLOCator [15], NTFD [16], or CompoundDiscoverer 3.x (Thermo Scientific). Each of these tools has been developed for particular isotope patterns or is based on direct comparison of native and labeling-derived isotope signatures and is therefore limited to its respective specific application. For example, MetExtract II [11] and HiTIME [13] both require LC-HRMS data sets consisting of native and uniformly 13C-labeled compounds and
thus detect either such labeled metabolites or their bio-
transformation products. On the other hand, X13CMS [12],
geoRge [14] or NTFD [16] detect compounds based on de-
vviations from native or natural isotopolog patterns after
reatment of biological systems with an isotopically labeled
precursor (such as U-13C6 glucose) in comparison to the
same samples treated with the non-labeled precursor. To
the best of the authors’ knowledge however, there is no soft-
ware tool available to date, that allows the user to freely and
flexibly specify a custom isotopolog pattern, which is then
searched for in the LC-HRMS data set. Here we present
Custom Pattern Extract (CPExtract) that allows the user to
specify custom isotopolog patterns together with pre-set
relative intensity ranges to be searched for, thereby ena-
bling to specifically filtering for metabolite ions of interest
that agree with the desired and diagnostic isotopolog pat-
ttern.

METHODS
1. CPExtract application example
The application of CPExtract is presented by searching spe-
cifically for metabolites of the malonate pathway, namely
fatty acids, polyketides, and possible hybrid metabolites
thereof (e.g. nonribosomal peptide-polyketides or prenyl-
ated polyketides) in culture samples of the filamentous
fungi F. graminearum and T. reesei.

Two complementary tracer approaches were used. First
1,2,3-13C3-malononic acid diethyl ester was used as tracer sub-
stance. This approach will henceforth be referred to as the
standard tracer approach, since the tracer was isotopically
labeled (13C) and the cultivation was carried out with native
glucose as the sole carbon source in the growth medium.

The second tracer substance was malonic acid monomethyl
ester. Since this substance is not readily available in a 13C
isotopically labeled form, a complementary approach was
chosen, which will henceforth be referred to as the reversed
tracer approach. Here the native form of malonic acid
monomethyl ester was used as a tracer, while the only other
available carbon source for the fungus was U-13C6-labeled
glucose. Detailed information about the cultivation work-
flow can be found in the supplementary information.

2. CPExtract algorithm
The automated CPExtract workflow is based on the MetEx-
tract II software, which originally has been designed to
detect pairs of native and uniformly 13C-labeled metabolite
ions. CPExtract replaces this fixed and rigid definition by a
set of freely definable rules by which the isotope patterns to
be searched for can be arbitrarily defined by the user. The
individual rule set is defined via a Python vector of objects
where each is an instance of one of the currently available
"Rule" objects, which include presence or absence of certain
isotopologs as well as abundance or area-ratios between
different isotopologs. Further custom user-defined rules
can be easily added by defining additional subclasses. Auto-
matic data processing then filters for all signals with isotope
patterns that correspond to the defined set of rules. A sche-
matic representation of the data processing workflow and
algorithm is shown in Figure 1.

The individual steps of the processing workflow and CPEx-
tract algorithms are:
1. In order to be processed the manufacturer-specific raw
data chromatograms must be converted into the
mzXML format with e.g. ProteoWizard MSConvert [17].
2. Data processing in CPExtract starts at the scan level,
whereby each individual m/z signal in a full scan mass
spectrum is initially considered to represent the principal
isotopolog of a putative metabolite ion of interest
(henceforth termed X). This assumption is then verified
by checking the user defined set of rules. Currently, the
following distinct rules are available: The “Pre-

cenceRule" (a particular isotopolog must be present and
be within expected ratios relative to other isotopologs)
and "AbsenceRule" (a specified isotopolog must not be
present). For these two rules a "minIntensity" parameter,
which can be used to define a minimum signal in-
tensity that the isotopolog must have and a "verify-
ChromPeaksSimilarity" parameter, which specifies that
the chromatographic peaks of the isotopologs must be
present and co-elute. Furthermore the "RatioRule" (checks
whether an intensity ratio of two isotopologs or
even two groups of several summed up isotopolog
intensities corresponds to a certain defined ratio or
checks if both ratios are within a defined range), the
"AnyIntensityRule" and the "AllIntensityRule" (either
any or all specified isotopologs must exceed a certain
intensity threshold) are available. Moreover, users can
easily implement their own rules if required. It is then
checked if the isotopologs defined in the rule set rela-
tive to the assumed X isotopolog are valid (e.g. if certain
isotopologs are present or absent). Only if X and all its
putatively related isotopolog peaks meet the specified
rules, X is used for subsequent data processing.
3. All MS signals (i.e., Xs) that fulfilled the rules in the previous step are then clustered using hierarchical clustering. Sub-clusters of the thereby generated dendrogram within a low mass deviation window (scan-to-scan variability) are kept. This dendrogram cutting step also takes the acquisition time (scan number according to the retention time) of the MS-scans into consideration so that isomers end up in different sub-clusters.

4. Data processing then continues on the chromatographic level. Therefore, an average m/z value is calculated for Xs of each sub-cluster present from the previous step, and peak picking is carried out using the continuous wavelet transform (CWT)-based centWave algorithm [18].

5. Only those isotope pattern candidates (or Xs) identified in the previous steps, whose chromatographic retention and peak shape show sufficient agreement with the user's rules, are ultimately accepted as hits. For this, all chromatographic peaks of the isotopologs must show perfect chromatographic co-elution, which is tested via the Pearson correlation coefficient, and additionally also the isotopolog ratios based on the chromatographic peak areas are again checked and must meet the user's rules-criteria.

6. The remaining steps of CPEXtract are the same as implemented in MetExtract II [11], namely bracketing of the detected features across all samples, chromatographic convolution (i.e. grouping and annotation of metabolite ions from the same metabolite), and peak area re-integration.

7. The results of the automated data processing are provided in the form of a tab-separated values (TSV) file containing a data matrix in which all detected principal isotopologs (Xs). The results can also be visualized and checked in the software's graphical user interface, whereby both the extracted ion chromatograms and the spectra are illustrated.

3. Fungal strains and cultivation conditions

_Fusarium graminearum_ PH-1 (wild-type) obtained from Gerhard Adam, BOKU, Institute for Microbial Genetics (IMIG) and three different _Trichoderma reesei_ strains, namely QM6aΔtms53 (wild-type) [19], QM6a Δtms53Δsor1 (a knock-out mutant lacking the gene encoding the SOR1 polyketide synthase, the first enzyme in the sorbicillinoid biosynthetic pathway) [20] and QM6a Δtms53Repr1 (a yellow pigment regulator 1 transcription factor over expressing strain) [21] were used for the preparation of culture medium supernatant and mycelium samples. To obtain the data sets, the samples were measured with LC-HRMS. Detailed information about the SIA-tracer workflow, cultivation, sample preparation and measurement are given in the Supporting information.

4. Conversion of LC-HRMS raw data files

The raw data files were converted into mzXML format using ProteoWizzard MSConvert Software [17] version 3.0.19210 (settings: mzXML output format, 32-bit binary encoding precision, enabled index writing, enabled TPP compatibility, disabled zlib and gzip compression, peak picking using vendor algorithm).

5. CPEXtract rules and parameter settings for data processing

5.1. Processing of LC-HRMS data generated with the standard tracer approach

The following set of rules was used to detect isotopolog patterns of fully 13C metabolites that incorporate several 13C units derived from the 13C-labeled malonic acid diethyl ester tracer as expected from the standard tracer approach:

X represents the native, monoisotopic form of the metabolite ions and consists only of 13C isotopes for each carbon atom and Xυ indicates that υ 13C atoms are exchanged for 12C atoms in that particular isotopolog relative to X.

- X must have a signal abundance of at least 1E5 counts. Xυ,2 and Xυ,4 must have a signal intensity of at least 5E4 counts. (part of PresenceRule)
- As X already represents the fully 13C isotope form of the metabolite, the signals for putative 12C-1 and 12C-2 isotopologs should therefore not be present. To avoid false negative results (e.g. signal noise), signals with an intensity value of maximum 5 % of X are accepted for both isotopologs without a rule violation. (AbsenceRule)
- The metabolite ion containing one and two 13C-malonic tracer derived extension units X2 and X4 must be present and their abundance has to be within the range of 10 to 300 % of X and X2 respectively. (RatioRule)
- The ratio of the abundance of X totaled with the abundance of Xυ,2 to the abundance of Xυ,1 must be the same as of the abundance ratio of Xυ,2 totaled with the abundance of Xυ,3 with a maximum permissible deviation of these ratios of ± 10 % respectively (i.e. (X+Xυ,2)/Xυ,1 = (Xυ,2+Xυ,3)/Xυ,3). (RatioRule)
- The isotopologs X, Xυ,1, Xυ,2, Xυ,3 and Xυ,4 must be present as co-eluting chromatographic peaks (Pearson correlation ≥ 0.85). (part of first PresenceRule)

5.2. Processing of LC-HRMS data generated with the reversed tracer approach

To detect isotopolog patterns of fully 12C labeled metabolites that incorporate one or more 13C units derived from the native malonic acid monomethyl ester, slightly modified rules compared to the standard tracer approach were used. In case of the reverse tracer approach, X represents the uniformly 12C-labeled form of the metabolite ions and consists only of 12C isotopes for each carbon atom and Xυ indicates that υ 13C atoms are exchanged for 12C atoms.

- X must have a signal abundance of at least 1E5 counts. Xυ,2, Xυ,4 and Xυ,6 must have a signal intensity of at least 5E4 counts. (PresenceRule)
- Since here X already represents the fully labeled 12C isotope form of the metabolite, the signals for putative 13C-1 and 13C-2 isotopologs should not be present. Signals up to 5 % of X were tolerated. (AbsenceRule)
- The metabolite ion containing one, two and three 13C-malonic tracer derived extension units Xυ,2, Xυ,4 and
X₄ must be present and their abundance has to be in a range of 10 to 300 % of X, X₂ and X₄ respectively. (RatioRule)

Moreover, the metabolite ions containing one or two extension units with one ¹³C and one ¹⁴C (i.e. X₁, X₃ and X₅) must be present and must have an abundance of 10 to 200 % relative to X and X₂, X₂ and X₄, and X₄ and X₆ respectively. (RatioRule)

- The ratio of the abundance of X totaled with the abundance of X₂ to the abundance of X₁ must be the same as of the abundance ratio of X₂ totaled with the abundance of X₄ to the abundance of X₃ as well as the abundance ratio of X₄ totaled with the abundance of X₆ to the abundance of X₅ with a maximum permissible deviation of these ratios of ± 10 % respectively (i.e. (X+X₂)/X₁ ≈ (X₂+X₄)/X₃ ≈ (X₄+X₆)/X₅). (RatioRule)
- All isotopologs defined via “PresenceRule” instructions (X, X₁, X₂, X₃, X₄, X₅ and X₆) had to be present as co-eluting chromatographic peaks and must show a highly similar peak shape (Pearson correlation ≥ 0.85). (part of first PresenceRule)

5.3. General processing parameters

The further processing parameter settings of CPExtract were: intra-scan-mass-accuracy: ±5 ppm, inter-scan-mass-deviation: ±8 ppm, EIC-extraction window: ±5 ppm, retention-time-window: 3-36 minutes, minimum maximum chromatographic peak width: 5-25 seconds, minimum Pearson correlation for co-eluting chromatographic peaks: 0.85, maximum allowed retention-time-deviation for bracketing of multiple measurements: 0.1 minutes, maximum allowed m/z deviation for bracketing: 5 ppm.

RESULTS AND DISCUSSION

A novel software tool, named CPExtract, for the comprehensive and automated search for isotope patterns in LC-HRMS data that can be freely defined by the user is presented. Other than existing software tools, it provides the user with an automated framework to mine the LC-HRMS data for certain characteristic isotopolog pattern via a set of predefined and extensible rules. Only isotopolog patterns obeying these rules are reported. Moreover, the large flexibility in defining the target patterns enables to use the CPExtract algorithm in two complementary ways, a standard tracer, as well as a reversed tracer approach. As a proof of concept, the CPExtract software was used to search specifically for fungal metabolites of the malonate pathway, namely fatty acids and polyketides as well as their derivatives like e.g. nonribosomal peptide-polyketide (NRP-PK) or prenyl-polyketide hybrids in LC-HRMS data.

1. Expected theoretical isotope patterns

1.1. Standard tracer approach

Figure 2 shows a typical theoretical isotopolog pattern of a substance which is to be expected for the standard tracer approach, due to the accidental incorporation of both added (¹³C) tracer-derived and native fungus-produced C₂ building blocks into its carbon skeleton. Such a pattern can be expected when the bioavailability and incorporation rate of tracer molecules is small in relation to the amount of malonyl-CoA units, endogenously formed from native glucose and used by the fungus itself for biosynthesis.

A staircase shaped isotope pattern is characteristic for continuously produced metabolites, corresponding to the decreasing probability that an increasing number of tracer-derived C₂ units become incorporated into the synthesized metabolite.

In addition, also the X₁₃, X₇ and X₁⁵ isotopologs are of high diagnostic value. They result only from the proportion of non-monoisotopic tracer derived extension units incorporated into the metabolites that occur due to the natural abundance of ¹³C isotopes in the native carbon source and the ¹²C isotopic impurity of the ¹³C labeled tracer compound used. Therefore, their signal intensities must be smaller than the mean value of the two neighboring isotopologs with even numbered mass increment (e.g. X₁₃ < (X+X₁₃)/2). Furthermore, the resulting intensity ratio (e.g. (X₁₃+X₁)/X₁) must be almost the same for all analogous isotopolog pairs (e.g. (X₂+X₂)/X₂). The intensity ratio of odd-numbered isotopologs to their even-numbered neighbors is independent of the number of tracer-derived extension units incorporated into the particular metabolite ion, making them ideal for highly specific filtering.
1.2. Reversed tracer approach

Often $^{13}$C-labeled substances are expensive or not available at all. In this case, the reversed approach can be a solution to the problem. When combined with global $^{13}$C labeling of the biological system, the use of a native tracer such as malonic acid monomethyl ester results in a typical isotope pattern that is a mirror image inversion of the pattern expected from the standard tracer approach (Figure 3).

Due to the high flexibility in defining rules for the isotope patterns to be searched for, the CPEXtract software is able to deal with both tracer approaches equally.

2. Exemplification of isotope pattern and tolerance intervals for oleic acid

Figures 4 and 5 show the spectra of oleic acid experimentally measured in $F$. graminearum mycelium extract samples together with the identified mono- and isotopic isotopolog (X) and the respective tracer-derived isotopologs exemplarily for standard tracer- and reversed tracer approach respectively. For $T$. reesei mycelium extract similar patterns were observed (data not shown). In both illustrations the red boxes show the required isotopologs as well as their permissible intensity ranges as defined in the respective rule set for the standard- and the reversed tracer approach.

As can be seen from the figures, the rules selected are suitable for the detection of the isotope patterns in the measurement data caused by the random incorporation of tracer-derived $C_2$ extension units. Due to the rapidly decreasing signal intensity of isotopologs with an increasing number of tracer derived extension units, not all theoretically existing isotopologs were detected.

For this reason, the rule set of the standard tracer approach required the presence and permissible intensity of at least two $C_2$ extension unit isotopologs ($X_{-2}$ and $X_{-3}$) as well as the isotopologs $X_{-1}$ and $X_{-3}$ in between for a hit. In addition, the intensity ratios of the sum of $X_{+2}$ to $X_{+1}$ and $X_{+2}$ plus $X_{+3}$ respectively had to be equal with a maximum permissible deviation of $\pm 10\%$.

With the reversed tracer approach more abundant signals for the $X_{+n}$ isotopolog signals ($X_{n}, X_{n+1}, \ldots$) were obtained compared to the standard tracer approach. Therefore, the rule set was extended by an additional pair of isotopologs corresponding to a third tracer-derived $C_2$ extension unit ($X_{+5}$ and $X_{+6}$), further increasing the specificity.

3. Isotope patterns found for the polyketides aurofusarin and sorbicillinol

The experimentally observed isotopolog patterns after data processing with CPEXtract are exemplified with the two known polyketides aurofusarin for $F$. graminearum and sorbicillinol for $T$. reesei. Results are shown for both tracer approaches in Figure 6.

The spectrum of aurofusarin from the reversed tracer approach showed all fifteen isotopolog signals corresponding to the incorporation of all fifteen possible malonate tracer-derived $C_2$ extension units into the carbon skeleton. In contrast, for aurofusarin in the standard tracer approach and for sorbicillinol in both approaches, only some of the theoretically possible isotopologs were sufficiently abundant for being detected.
While the large tolerance windows needed for the initial pattern search can potentially compromise the filtering specificity and thus lead to false positive search results, additional pattern shape characteristics can be used for further filtering of candidate ions.

Insufficient predictability of the overall shape of the pattern does not affect the respective intensities of the isotopologs located between two adjacent even-numbered isotopologs (e.g. for X-1 between X and X-2). As already explained above, the odd-numbered (X+1, X+3, ...) isotopologs always result from the sum of the respective isotope fractions and the number of carbon atoms in the metabolite. Furthermore, and perfectly usable as a reliable selection criterion, the relative ratio of the intensity of these isotopologs to that of the respective two analogous neighboring isotopologs with a differing number of tracer derived extension units must remain the same. Therefore, much smaller tolerable intensity intervals can be defined in the rules for the odd-numbered isotopologs without running the risk to miss target metabolite ions. To this end, the initially created candidate list can be refined by checking the constant relative intensities as described above to sort out remaining false positives very reliably.

5. Global screening of all tracer-derived metabolite ions

The big advantage of filtering MS features according to certain metabolite classes by CPEXtract using specific isotope patterns derived from the corresponding tracer can clearly be seen in Figure 8.

In the F. graminearum data set, the MetExtract II evaluation of the global labeling approach resulted in a total number of 621 metabolites found both in supernatant and mycelium extract samples of the wild-type strain PH1 that were actually produced by the fungus (Figure 8). The CPEXtract algorithm detected a subset of tracer-derived metabolites from this set, comprising 182 metabolites for the standard tracer approach and 191 metabolites for the reverse tracer approach. This clearly demonstrates the filter effect of the tracer approach combined with the automated CPEXtract data evaluation on the targeted metabolic pathways.

In the T. reesei data set, consisting of supernatant and mycelium extract samples of each of the three different strains,
1165 metabolites truly originating from the fungus were found by MetExtract II data evaluation. The CPEXtract algorithm data processing yielded 167 tracer-tracer-derived metabolites for the standard tracer approach and 283 for the reversed tracer approach respectively (Figure 8).

The relatively high number of (malonate derived) metabolites can be explained on the one hand by the combination of three different fungal genotypes and on the other hand presumably due to the high reactivity of the sorbicillinoids. Members of this class of polyketides are known to spontaneously react non-enzymatically into various other substances or reaction by-products. Figure 9 shows for both tracer approaches all found feature groups detected in the T. reesei culture samples. In the Venn diagrams it can be seen that no metabolites annotated as sorbicillinoids were found in the ∆Sor1 strain samples, which is as expected since this strain is deficient in the sorbicillinoid polyketide gene cluster and thus not able to produce any sorbicillinoids at all. Furthermore, it can be seen that some additional sorbicillinoids (namely bisorbidicinol, epoxysorbicollinol, sorbiquinol, bisvertinolone, dihydrobisorvicinol) were found exclusively in samples of the overexpressing ReYpr1 strain.

![Figure 9](image_url)

**Figure 9** | Venn diagrams of all metabolites found with both, the standard tracer and the reversed tracer approach in the investigated T. reesei strain culture samples (wildtype (WT) in green, Ypr1-overexpressing ReYpr1 strain in red and ∆sor1 deletion strain in grey). The annotated sorbicillinoids are shown in yellow.

6. Selectivity of CPEXtract algorithm

Furthermore, to test the selectivity of the CPEXtract algorithm and the rule sets used, culture samples without the addition of the tracer compounds were measured and processed using the same settings. This was done for both the standard- (13C5 glucose) and reversed (13C6-glucose) approach. In these negative control samples a low number of hits (false-positives) were detected by CPEXtract, demonstrating the high specificity of the presented approach. Surprisingly, only in some T. reesei negative control samples corresponding tracer-derived isotope patterns were detected. In total, seven corresponding tracer-derived patterns (different ones in each replicate) were detected in supernatant samples of the Ypr1 overexpressing ReYpr1 strain and one tracer-derived pattern was detected in a single replicate of the WT supernatant samples. Manual inspection of the raw data files revealed that the false positive signals were only detected immediately after the measurement of samples, which showed high signal intensities for exactly those ion species. Therefore, it was concluded that the false positives resulted from carryover into successive injections via the insufficiently rinsed injection needle of the HPLC system. Thus, the false positive hits were not caused by a low selectivity of the CPEXtract algorithm but correctly detected true polyketides (data not shown).

**CONCLUDING REMARKS**

Based on the exemplary search for metabolites of the malonate pathway in the culture samples of two different filamentous fungi using two complementary tracer approaches, it was possible to demonstrate the reliable function of the CPEXtract algorithm and its universal and versatile applicability. Through the detection of unknown metabolites, specifically from the substance group of interest, in addition to the known and expected metabolites of the respective fungus, it is evident that the CPEXtract software can also benefit the search of various other putatively novel or even completely unknown metabolites. Especially the timely but still demanding topic of natural product discovery is a promising field of application of CPEXtract. As exemplified in this article, the reliable filtering the MS data for the fatty acids and polyketides searched for, enables a drastic reduction in the complexity of the global metabolite profiles. This can help to greatly simplify manual data curation that is subsequently required.

CPEXtract shows also great potential for other natural product classes such as nonribosomal peptides or terpenoids when being combined with other tracer compounds such as amino acids or mevalonate. Another interesting option is to screen for functionalization of natural products like for example methylation by the use of L-methionine-(methyl-13C). Last but not least the metabolic fate of (labeled) potentially toxic xenobiotics can be probed by CPEXtract either with the standard or reverse approach.

We anticipate that the presented tracer-approach in combination with the flexible and easy-to-use CPEXtract software will be of interest to the metabolomics community working in related fields of research.

CPEXtract is available free of charge for non-commercial use at [https://metabolomics-ifa.boku.ac.at/CPExtract](https://metabolomics-ifa.boku.ac.at/CPExtract). Likewise, the LC-HRMS data can be obtained from [https://metabolomics-ifa.boku.ac.at/CPExtract/#datasets](https://metabolomics-ifa.boku.ac.at/CPExtract/#datasets).

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website. One section with additional text and three figures describing the SIA-tracer workflow, cultivation, sample preparation and measurement in detail and showing screenshots of CPEXtract software (PDF).

**AUTHOR INFORMATION**

**Corresponding Author**

* Corresponding author (rainer.schuhmacher@boku.ac.at)

**Author Contributions**

BS and RS designed the experiments. BS carried out the biological experiments and the LC-HRMS analysis. BS and CB implemented the software tool and processed the data set. All authors contributed to the biological interpretation and the implementation of the manuscript.

**Notes**

The authors declare no conflict of interest.
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