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How to screen and identify unexpected and unwanted compounds in food

Authors

Malin Johansson,
Student in the Master's program of Analytical Chemistry at Uppsala University, Sweden

Susanne Ekroth,
Chemist at the National Food Agency, Uppsala, Sweden

Olaf Scheibner,
Thermo Fisher Scientific, Germany

Maciej Bromirski,
Thermo Fisher Scientific, USA

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Goal

To develop a non-targeted workflow for Thermo Scientific™ Compound Discoverer™ software to screen for unexpected and unwanted substances in food.

Introduction

The laboratories of food safety authorities are faced with many challenges in screening for an ever-increasing number of analytes, both expected as well as unexpected. It is crucial to have a generic multiresidue method that can handle the wide range of polar and non-polar analytes as well as the complexity of food matrices. Approaches such as SweEt (Swedish Ethyl acetate method)[1] enable and facilitate fast and easy sample extraction of foods high in moisture, sugar, acid, oil, or starch faster and more straightforward. One of the most unique features of the SweEt method, developed for pesticides at the Swedish National Food Agency, is that it involves a single-step ethyl acetate extraction procedure, after which the sample can be injected directly (with no solvent exchange) into the instrument of analysis. Pesticides alone account for around 1000 analytes and can be found in the food commodities found in Figure 1. Meanwhile other types

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of unwanted analytes can also be found such as mycotoxins, alkaloids, food additives, veterinary drugs, and food contact materials. With the high specificity, sensitivity, and accuracy of an HRMS Orbitrap™ instrument, simultaneous screening and identification of multiple analytes are enabled in one run. Full scan with data-dependent MS^2 ($ddMS^2$) acquisition has proved to be practical for the quantification and identification of target compounds and detection of non-target compounds in the same workflow.

The key to successful identification is the availability of powerful software that can provide automated and reliable detection of candidate compounds. This can be performed with Compound Discoverer software, which also evaluates them statistically. With Compound Discoverer software it is possible to evaluate mass spectral data, predict the elemental composition, and search databases for possible identities of candidate compounds.

Experimental

Sample preparation

Figure 2 shows the workflow for the accredited SwEt multiresidue method[1] with the number of validated pesticides above each group. This method is used for analyzing pesticides routinely in official control, using both GC-MS/MS and LC-MS/MS, and has been shown to be a generic multiresidue method for analyzing different compound classes.

Liquid chromatography method

The Thermo Scientific™ Dionex™ UltiMate™ 3000 standard system was used as an HPLC system. The details of the chromatographic settings used are shown in Table 1.

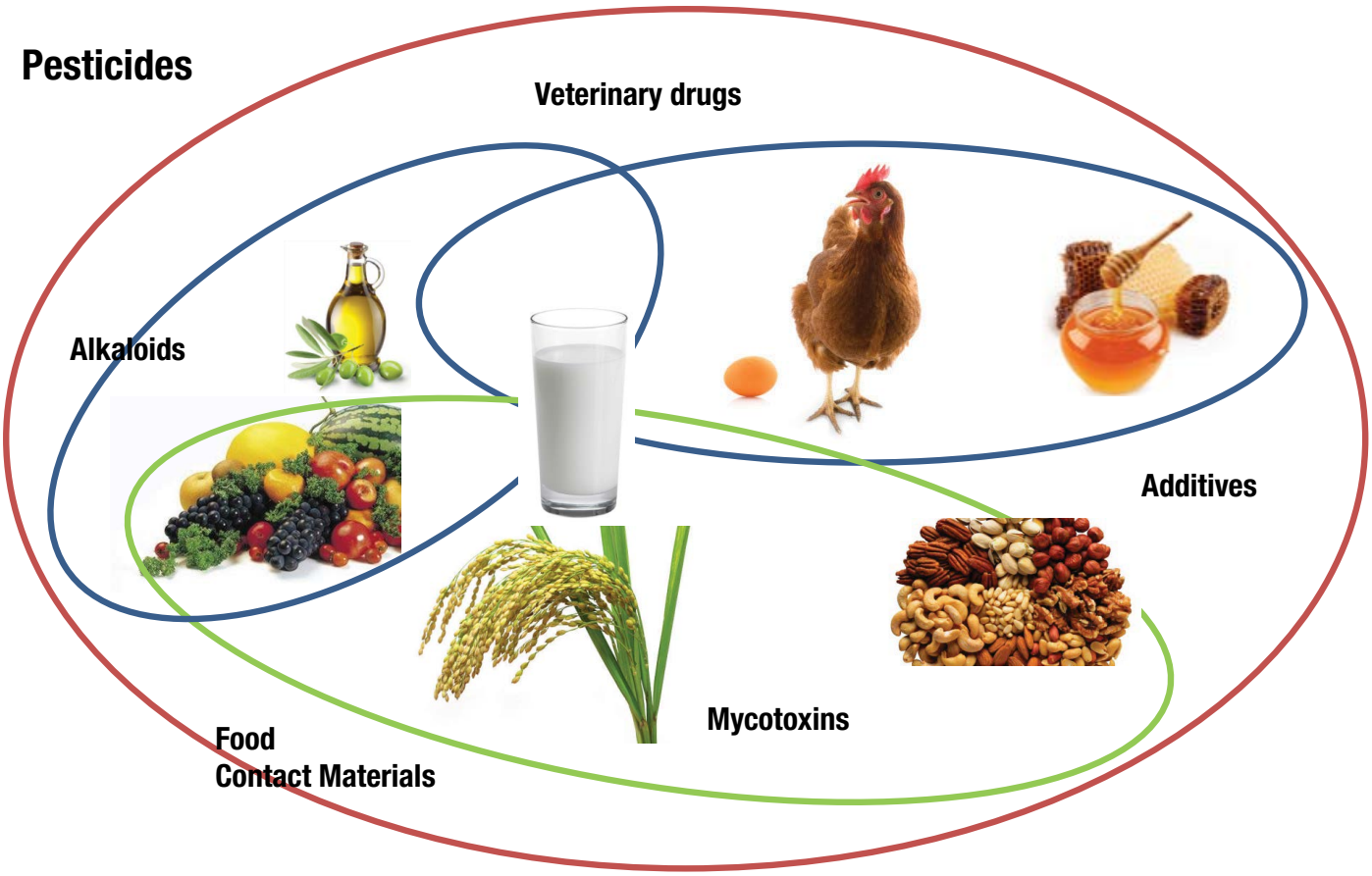


Figure 1. Example of possible combinations for screening several different compound classes, potentially present in samples.

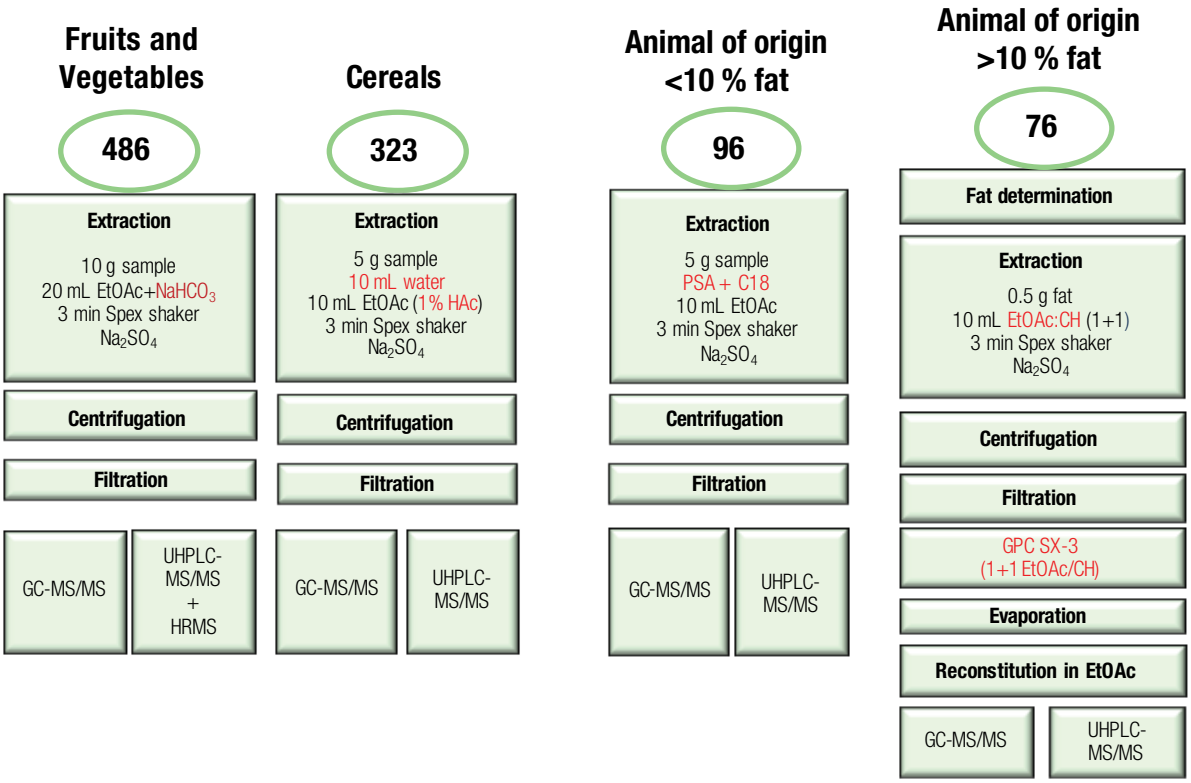


Figure 2. The SweEt workflow for different commodity groups.

Table 1. LC settings.

Instrumentation:	Dionex UltiMate 3000 standard system
Column:	Thermo Scientific™ Accucore™ C18, 100 × 2.1 mm, 2.6 µm particle size
Column temperature:	30 °C
Mobile phase A:	Milli-Q® water with 5 mM ammonium formate and 0.1% formic acid
Mobile phase B:	MeOH with 5 mM ammonium formate and 0.1% formic acid
Gradient:	Mobile phase B changes accordingly: 12 min 0 to 100% 4 min of 100% 1 min 100 to 0% 1 min 0 to 100% 1 min 100 to 0% 1 min 0%
Flow rate:	0.3 mL/min
Injection volume:	2 µL

Mass spectrometry method

The Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap™ mass spectrometer in full scan with data-dependent MS^2 was used in Discovery mode (Figure 3). The properties of the $ddMS^2$ experiment are shown in Table 2.

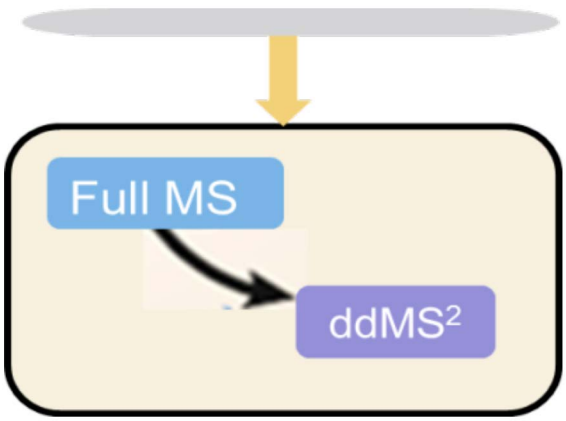


Figure 3. Scan mode.

Table 2. MS settings.

Instrumentation:	Q Exactive Focus MS
Full Scan	
Resolution setting:	70,000 (FWHM) at *m/z* 200
Scan range (*m/z*):	120–1000
ddMS²	
Resolution setting:	17,500 (FWHM) at *m/z* 200
Isolation window (*m/z*):	1.5
Spray voltage:	3.60 kV
Sheath gas:	7.0
Aux gas:	0.0
Capillary temp.:	320 °C
Heater temp.:	0 °C
RF-lens level:	50
HCD collision energy (stepped):	ce: 20, 80 eV

Compound Discoverer software

Thermo Scientific Compound Discoverer software is a comprehensive and efficient software tool that uses isotopic pattern recognition, fragments, and isotope distribution to generate confirmation of identity. The generation of elemental composition uses isotopes, accurate mass, and fragments to generate match scores. The MS^2 data produced in the collision cell can be used for spectral searches in the mzCloud™[2] and Thermo Scientific™ mzVault™[3] environments. The ChemSpider database is used for parent mass and elemental composition searches.

A workflow is created by connecting nodes containing parameters that are adjusted to obtain optimal results (Figure 4).

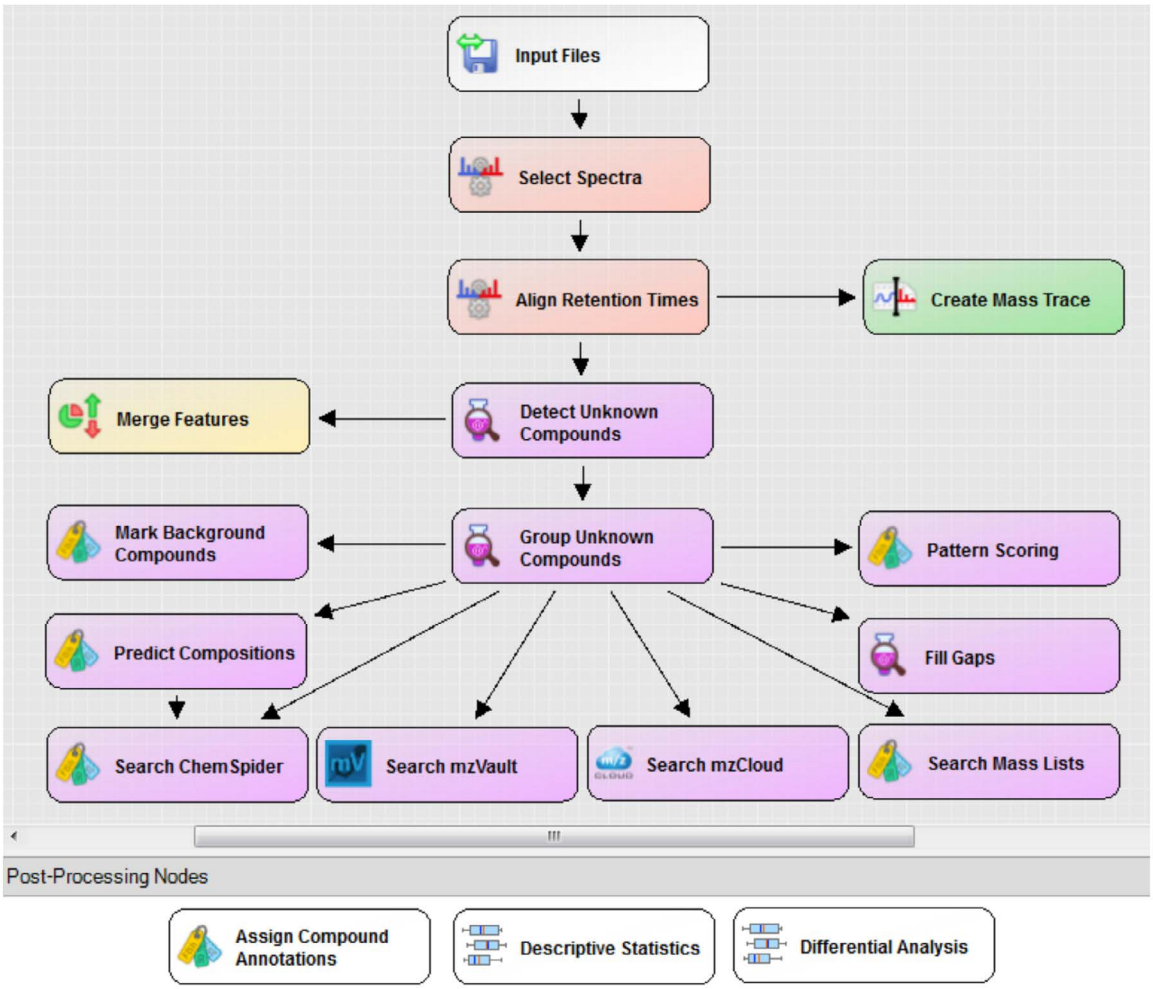


Figure 4. Workflow for non-targeted screening.

The most important parameters in the workflow are set in the Detect Unknown Compounds node, including elemental composition, adducts, mass tolerance, and intensity threshold (Figure 5).

1. General Settings	
Mass Tolerance [ppm]	2 ppm
Intensity Tolerance [%]	30
S/N Threshold	3
Min. Peak Intensity	500000
Ions	[M+H]+1; [M+K]+1; [M+Na]+1; [M-H]-1
Base Ions	[M+H]+1; [M-H]-1
Min. Element Counts	C H
Max. Element Counts	C90 H190 Br3 Cl4 K2 N10 Na2 O18 P3 S5
2. Peak Detection	
Filter Peaks	True
Max. Peak Width [min]	0.8
Remove Singlets	True
Min. # Scans per Peak	5
Min. # Isotopes	1

Figure 5. Parameters of Detected Unknown Compounds node.

Spectral databases

mzVault software is used to search libraries of accurate mass data for desired spectrum or spectra. It is possible to customize the library by adding or deleting spectra and opening and creating libraries.

The library of mzCloud (developed by HighChem LLC) is a free, online, and open access database, continuously updated with new compounds. The final matching results generated using the mzVault and mzCloud libraries provide compound name, structure, molecular formula, molecular weight, and most importantly, a match score.

ChemSpider

The ChemSpider databases chosen for the experiment are listed in Table 3. It is important to choose relevant databases, but also to take into consideration their size to make the search feasible. Even though the databases are large, the results are regulated in the workflow where the maximum number of potential matches is set.

Mass lists

Local databases are used to predict name, molecular formula, and molecular weight. It is possible to use several databases, both databases provided by Thermo Scientific as well as those developed in the laboratory.

Table 3. List of ChemSpider databases used.

Data Source	Count	Description
ACToR: Aggregated Computational Toxicology Resource	96001	Industrial chemicals, pesticides, water contaminants
DrugBank	7082	Drugs
EPA DSSTox	671300	Toxicity data of chemicals
FDA UNII – NLM	61801	Identifiers for substances in regulated products
Food and Agriculture Organization of the United Nations	1453	Food additives, contaminants, naturally occurring toxicants, veterinary drug residues
FooDB	16744	Food nutrients
LeadScope	140254	Toxicity data of chemicals
Lhasa Limited	9271	Toxicity data of chemicals
Pesticide Common Names	1560	Pesticides
Wikipedia	11882	Encyclopedia

Results and discussion

The chromatogram shown in Figure 6 confirms that the SweEt multiresidue method is an effective generic method to analyze different compound classes. The following were found in basmati rice:

Pesticides

- Buprofezin
- Triazophos
- Tricyclazole

Mycotoxins

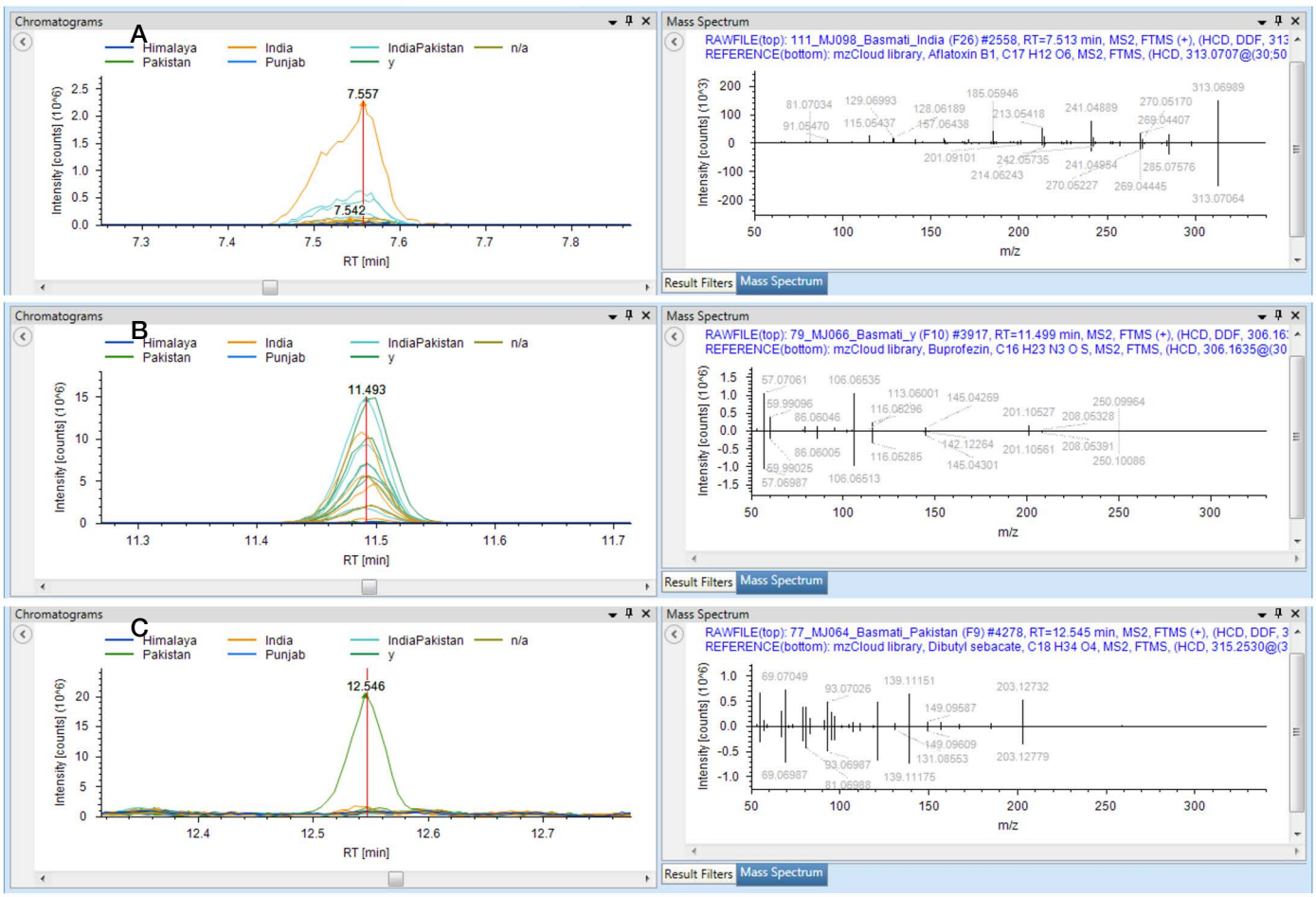
- Aflatoxin B1

Plasticizer/Food Additive

- Dibutyl sebacate

The identities of the pesticides and aflatoxin B were confirmed with standards, but the compounds were not quantified, as this was not the aim of this project. Because of time constraints, no standard for dibutyl sebacate was available.

There can be several causes for the finding of dibutyl sebacate. Its use as a food additive and as a plasticizer is authorized, as well as its use in food package gaskets.[4] However, it might be of special interest regarding food fraud since fake grains made by plastic have been found in rice that can enhance the weight and volume.[5] Similar findings with pesticides and aflatoxins were found in figs. The final results from 28 rice samples generated 65,000 features. Therefore, it is very important to have an efficient filtration mechanism in the processing software to reach the relevant results.



Checked	Name	Formula	Annotation	Molecular Weight	RT [min]	Area (Max.)	# ChemSpider Results	mzCloud Best Match	mzVault Best Match	Mass List Matches
☑	Dibutyl sebacate	C18 H34 O4		314.24544	12.547	789861	54	92.9	89.7	
☑	Aflatoxin B1	C17 H12 O6		312.06303	7.558	104551	37	85.7	76.2	
☑	Buprofezin	C16 H23 N3 O S		305.15576	11.492	661526	4	83.4	92.7	
☑	Tricyclazole	C9 H7 N3 S		189.03587	6.458	636074	27	82.1	84.7	
☑	Triazophos	C12 H16 N3 O3 P S		313.06455	9.935	435156	1	81.4	87.4	

Figure 6. Example of LC-MS chromatogram, mass spectrum, and results table from positive findings in rice: (A) aflatoxin B1, (B) buprofezin, (C) dibutyl sebacate.

The results of the rice samples are displayed in the PCA plot in Figure 7. The sample containing dibutyl sebacate is differentiated from the other rice samples as it appears farthest to the left in the plot. This difference does not only depend on the dibutyl sebacate but also of all non-identified features that are generated in the analyses.

The PCA plot indicates that the rice sample differs in composition from the others. To be able to identify the other components in the sample, a non-targeted approach is needed since only the elemental formula is generated for those features.

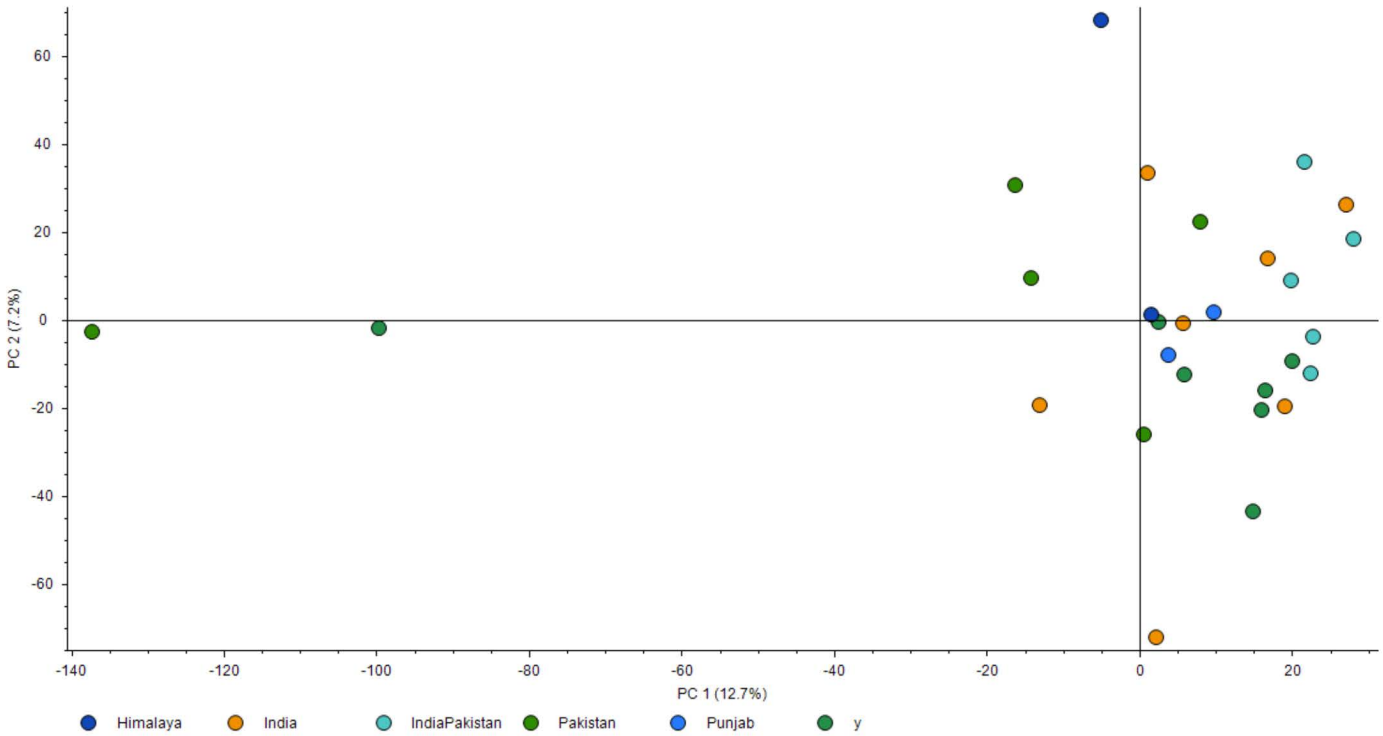


Figure 7. PCA plot showing rice samples. The sample farthest to the left is the sample containing dibutyl sebacate.

This study was conducted using fruits, vegetables, nuts, and cereals using the appropriate SweEt extraction method (Figure 2). Similar workflows will be used in the future to find other analytes, for example veterinary drugs together with additives, mycotoxins, and pesticides in animal origin products (Figure 1).

In addition to identification of compounds, Compound Discoverer software also provides a statistical evaluation, which can be used to show similarities as well as diversities of different commodities. These can be illustrated with PCA plots, volcano plots, and trend charts. The PCA plot in Figure 8 shows 25 samples grouped into five separate commodity groups, except for some overlapping between avocado and melon. In the future, information about different species within one commodity group will be investigated since it could be an important parameter when interpreting grouping in the PCA plot.

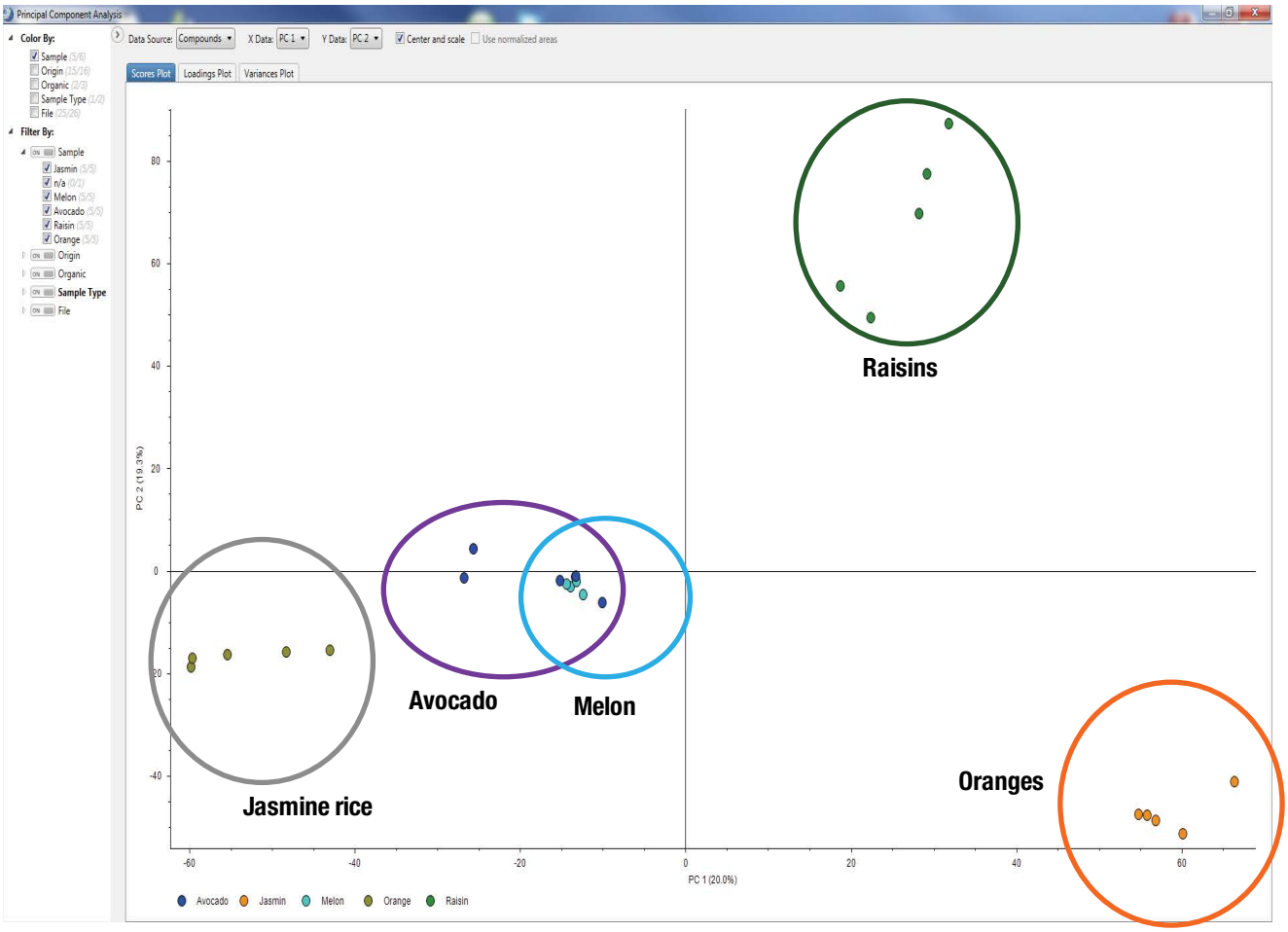


Figure 8. Clustering of different commodity groups.

Conclusion

A screening method was developed to find unexpected and unwanted analytes for several compound classes in the same matrix as well as making food control even more efficient in the future. This was done in combination with Compound Discoverer software, a powerful qualitative tool, that provides confident results with several identification parameters for unknown compounds.

Compound Discoverer software offers interesting statistical evaluation where it is possible—for example, to group compounds of different commodities using a PCA-plot, filter results according to p-value, or to quickly identify changes in large data sets using the volcano plot.

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