Evaluation and validation of an analytical approach for high-throughput metabolomic fingerprinting using direct introduction–high-resolution mass spectrometry: Applicability to classification of urine of scrapie-infected ewes

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Evaluation of direct introduction-high resolution mass spectrometry (DI-HRMS) based metabolomics approach for the discrimination of several exposure groups to pesticides: comparison with liquid chromatography high resolution mass spectrometry (LC/HRMS)

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Abstract

One of the current public health concerns is the evaluation of population exposure to toxicants present in food and environment. To do this, high-throughput methods are required to perform a large number of analyses. The aim of this work is to characterize human exposure to xenobiotics. The classification of urines from farmers occupationally exposed to five pesticides was investigated using two analytical techniques, *i.e.* the

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classical approach based on liquid chromatography coupled to high resolution mass spectrometry (LC/HRMS) and direct introduction-high resolution mass spectrometry (DI-HRMS) to produce metabolomics data. Discrimination of different exposure groups was obtained from LC/MS data as well as from DI-HRMS data thanks to the Independent Component - Discriminant Analysis (IC-DA). The DIMS approach requiring only a fraction of the LC/MS analysis time, appears to be very promising for high throughput epidemiological studies.

Keywords: Metabolomics, Pesticide exposure, Direct introduction mass spectrometry, Liquid chromatography mass spectrometry, High resolution mass spectrometry, Independent component - discriminant analysis.

1. Introduction

The presence of toxicants (*e.g.,* pesticides, drugs, pollutants or contaminants) in food and environment can impact human health. Due to the great diversity of xenobiotics and the complexity to develop targeted methods specific to a molecule or a compound family, global approaches without a priori such as metabolomics seem to be appropriate in such purpose. Metabolomics [1] is the study of small molecular mass metabolites found in biological samples, which are involved in growth, metabolic homeostasis or etiology of disease. It aims at the detection, quantification and subsequent identification of different metabolic changes arising between two (or more) well-defined but contrasted situations (*e.g.*, healthy *versus* different unhealthy status). Analytical techniques combined with statistical and/or chemometric processing tools are essential to compare generated

metabolomic fingerprints reflecting phenotypic differences and to reveal the metabolic biomarkers of a given physiological condition or a pathological state [2].

Among the available analytical technologies, mass spectrometry (MS) providing high sensitivity, specificity and selectivity, and especially, Fourier transform mass spectrometry (FTMS) with its very high mass resolving power, high mass measurement accuracy and wide dynamic range should produce high quality of metabolomic profiles in term of isobaric species distinction and number of detectable peaks [3,4]. MS-based metabolomic analysis commonly uses hyphenated methods, typically liquid or gas chromatography coupled to mass spectrometry (LC/MS or GC/MS). The hyphenated approaches have the main advantages of being able to separate isobaric and some isomeric compounds and also to minimize the so-called matrix effects. They should enhance the metabolome coverage [5,6]. However, they cannot be really considered as high throughput approaches because of the analysis time. Although the use of ultra high performance liquid chromatography (UHPLC) can reduce the data acquisition time, its coupling to the FTMS is done at the expense of the mass resolving power. Furthermore, the pre-processing of the anisotropic data generated from several scans acquired along the chromatographic separation requires dedicated pretreatment steps such as peak alignment and correction for analytical drifts (*e.g.,* retention time and peak intensity). Alternatively, the direct introduction mass spectrometry (DIMS) approach enabling simultaneous detection of all ionized compounds provides a seemingly isotropic spectral fingerprint in a few minutes. Such direct approach can therefore be proposed to increase the throughput of analyses [7–9]. A comparison between DIMS and LC/MS approaches has been reported for the analysis of serum samples from kidney cancer patients [10]. A comparable group

classification was obtained from both approaches despite the smaller number of metabolites detected from DIMS data (*i.e.* 23 metabolites compared to a total number of 48 ions from LC/MS data). In addition, only a small fraction of the LC/MS analysis time (5%) is required to perform the DIMS experiment. Unfortunately, the DIMS approach using electrospray ionization (ESI) has some limitations, especially the occurrence of matrix effects. Such phenomena are characterized by ion suppression and/or signal enhancement, leading to unpredictable signal changes that may affect the quantitative response of the sample constituents and consequently modify the mass spectrum profiles [11]. These effects should be overcome or at least minimized through the optimization of analytical conditions. Sample dilution can be proposed to obtain metabolomics fingerprints containing the most informative data with minimized matrix effects without loss of peak intensity [12]. The use of high resolution mass spectrometry (HRMS) such as FTMS can compensate the absence of chromatographic separation by distinguishing compounds based on accurate m/z values and isobar ion resolution. Nevertheless, isomer distinction requires additional experiments such as the chromatographic separation and/or MS/MS experiments or other alternative technologies, such as the ion mobility spectrometry.

In our previous work, an in-house computational procedure was developed to process DI-HRMS data using a new discriminant analysis tool based on Independent Component - Discriminant Analysis (IC-DA). Successfully discrimination of farmers occupationally exposed to two types of pesticides was obtained, this constitutes the proof of concept of our approach for high-throughput metabolomics [13].

Most of metabolomic studies concern only few sample groups, typically healthy subjects *versus* one (or two) group(s) of unhealthy subjects [9,14,15]. Few investigations involving several classes have been reported, *e.g.*, in the context of dose [16] and time effects [17,18], but rarely in the context of human exposure during their lifestyle [19]. The main objective of this study is to demonstrate the applicability of our high throughput approach to classify several exposure groups. In addition to DI-HRMS, the hyphenated LC/MS technique was also employed to phenotype urine samples coming from farmers occupationally exposed to five different pesticides. Discrimination of the different exposure groups was obtained from both methods, highlighting the potential of the direct approach to provide satisfactory results comparable to those obtained by the LC/MS approach but with a significant gain in the analysis time.

2. Experimental

2.1. Materials

Ultra pure water was produced from a Milli-Q system (Merck Millipore, Guyancourt, France). The highest commercial grade of acetonitrile and formic acid was obtained from Sigma Aldrich Chemicals (L'Isle d'Abeau-Chesnes, France). Urine samples were provided by UMR1086 Cancers and Preventions. They were collected from 41 farmers before and after one day of occupational exposure to pesticides in the cultivated fields. The exposure conditions have been described by Lebailly *et al*. in a previous work [20,21]. There are five groups with exposure to: i) isoproturon (with n = 6 individuals), ii) captan (n = 16), iii) cyprodinyl (n = 7), iv) chlorothalonil (n = 8) and v) epoxiconazole (n = 4). Note that self-control samples (n=41) are available and correspond to urine of the same

subjects collected one-day before pesticide exposure. Note that all procedures performed in this work were in accordance with the ethical standards of the national research committee and with the 1964 Helsinki declaration and its later amendments. The collected urines were stored at -20°C until MS analysis. Urine sample normalization was done based on the creatinine concentration. Finally, urine samples were diluted at the chosen dilution factor of 500 after testing various dilution factors. Additionally, a quality control sample (QC, a pool of all urine samples) was injected after every set of 10 sample analyses to check for eventual analytical drifts and for the quality of the generated spectral data.

2.2. Mass spectrometry detection

A hybrid LTQ-Orbitrap Fourier transform mass spectrometer (LTQ Orbitrap XL, Thermo Fisher Scientific, Bremen, Germany) operating in positive ion mode was used to perform both DIMS and LC/MS analyses. In the DIMS approach, the following ESI conditions were used: electrospray voltage was set at -3 kV, capillary voltage at 13 V and tube lens offset at 60 V. Sheath gas flow and auxiliary gas were fixed at 70 and 5 arbitrary units (a.u.), respectively. The capillary temperature was maintained at 275 ˚C.

Mass spectra were acquired in the m/z 50-1,000 range with a cycle time of 4 minutes per analysis. A theoretical mass resolving power of 100,000 FWHM (Full width at half maximum) at m/z 400 was used. Internal mass calibration was performed using mass lock option on the $[M + H]^+$ contaminant ions of N-butylbenzene sulfonamide at m/z 214,089625 for both DIMS and LC/MS experiments. Flow injection analysis (FIA) mode was used to perform DIMS analyses. 10 µL of each 500-fold diluted sample were injected into a 100 µL sample loop using a Surveyor LC chromatographic system

(Thermo Fisher) at a flow rate of 30 µL/min of an acetonitrile/water mixture (1:1, v/v) plus 0.1 % formic acid as eluent. Tandem mass spectrometry (MS/MS) experiments were performed on the monoisotopic peaks of some metabolite species. The collision-induced dissociation (CID) conditions, the chromatographic separation and mass spectrometry conditions used for LC/MS analyses are described in the Supporting Information.

2.3. Data analysis

2.3.1 Data pre-processing

An in-house DIMS data processing procedure using Matlab routines (Matlab R2008a, The MathWorks, Natick MA, USA) was developed in our previous work [13] and was used in this study to create the DIMS data matrix. Alternatively, the LC/MS data were pre-processed using XCMS tool based on a program written in R (*R version 3.4.0, The R Foundation for Statistical Computing, Vienna 2017,* https://www.r-project.org) as described in the Supporting Information. The final number of extracted variables for the 82 urine samples was 747 and 2106 from DIMS and LC/MS data, respectively.

2.3.2. Chemometric methods

Multivariate analyses were further performed identically on both DIMS and LC/MS datasets, including all groups in a single model using two Matlab-based chemometric tools, as already described [13]. Briefly, the first preprocessing tool is the orthogonalization part of the OPLS algorithm [22]. The second tool is a supervised version of the Independent Component Analysis (ICA), named IC-DA, as previously described [13]. ICA aims to extract independent vectors, called Independent Components (ICs), corresponding to 'source signals' with true physico-chemical meaning [23–25]. IC-DA includes group membership in its calculation to extract signals that discriminate predefined groups.

Therefore, a second calculation was performed using only the spectral signals that contributed to group discrimination to evaluate if the given group is still separated. Nevertheless, because of the unbalanced classes in our case, group discrimination can be oriented by those containing more individuals. To avoid eventual overfitting, a validation step was then performed using a permutation procedure described in the previous work [13].

2.3.3. Selection and annotation of metabolic biomarkers

A quantile-quantile (Q–Q) plot was used to visualize variables that contribute most to group discrimination [26]. The most discriminant variables are characterized by high or low standard deviation values which depart from that linearity while non-interesting variables that follow a normal distribution are aligned. For each IC, only ions with an absolute intensity greater than 3 times the standard deviation of all the ions were selected. Alternatively, an additional procedure was performed to validate the selected discriminant variables, as well as to highlight their contribution to group discrimination. In this way, a random selection of 50 % of the individuals from each group, followed by IC-DA calculations was performed and repeated 3,000 times for every IC. Then, a quantile calculation with 95 % confidence limit was done on the generated matrix (variable number × 5 ICs × 3,000 repetitions). The discriminant variables characterized by a quantile sign (*i.e.*, positive or negative) were selected based on their high standard deviation values. The quantile sign of some selected variables changed after permutation. Those variables were then removed from the discriminant variable list.

Two computational tools such as the box-whisker plot and the Student's t-test were applied to highlight the contribution of a given variable to the discrimination of one (or several) exposure group(s). Box-whisker plots should show the abundance variations of a

given discriminant variable among all groups, including controls. However, these variations between groups were not obvious. Therefore, Student's paired t-tests were also done to reveal variable intensity variations before (self-control) and after exposure for every individual. The paired data structure was used here only to validate discriminant variables but it was not applied to all data because rarely each subject could be its own control (*e.g.*, self-control) in general population monitoring.

Afterwards, correlation coefficients were calculated between every selected discriminant variable and all other (discriminant or not) variables present in the mass spectra in order to find possible ion associations, *i.e.*, isotopic peaks, in-source fragments and/or adduct species generated from the same molecule. This calculation improves the compound annotation and can also reveal eventual biological relations between metabolites (see results and discussion). Finally, the most discriminant and correlated variables were putatively annotated using freely available databases (DBs) such as the Human Metabolome Database (HMDB) [27], the Kyoto Encyclopedia of Genes and Genomes database (KEGG) [28] and the METLIN [29] database. The experimental accurate m/z values with four digits after the decimal point were used to query DBs with a mass error below 5 ppm, except for ions with m/z values lower than 130 u, where 10 ppm mass error was used to find candidate compounds.

3. Results and discussion

3.1. Exposure group discrimination

Efficient discrimination of control and pesticide-exposed groups was obtained from both DIMS and LC/MS data using IC-DA (**Figs. S-1** and **S-2** in the supplementary materials).

A given group is typically discriminated from the others by a specific IC. However, these initial discriminations being obtained taken into account information on the group membership (*i.e.* dummy variables) are overoptimistic. New calculations only based on the MS signals contributing to each specific group discrimination, still provide distinction of all groups as shown in **Fig. 1** and **Fig. S-3** (supplementary materials) for DIMS and LC/MS, respectively. For the DIMS data, a slight overlap between control subjects and other groups is observed (**Fig. 1a**). Nevertheless, efficient separation is obtained for captan, chlorothalonil, cyprodinil and isoproturon exposure groups (**Figs. 1b-e**, respectively). Additionally, discrimination of the epoxiconazol-exposed group is visible in another projection dimension, i.e. IC2, IC3 and IC5 (**Fig. 1b-e**). A comparable group discrimination was obtained from LC/MS data (**Fig. S-3**) but the separation of control subjects from others is less pronounced compared to that for DIMS data (**Fig. S-3a**). Moreover, no clear distinction of the epoxiconazol-exposed group was obtained from the LC/MS data.

The 3D-plots (IC2 *vs* IC3 *vs* IC4) from calculations without the group membership information showed good discrimination of the four exposure groups, *i.e.* those exposed to captan, chlorothalonil, cyprodinyl, and epoxiconazol for both DIMS and LC/MS data (**Fig. 2**). Note that the samples from control group are located in the center of the plot and overlapped with the isoproturon-exposed group into the reported 3D plots. These two groups were separated in another dimension (**Fig. S-4** in the supplementary materials).

Hence, data generated from both the DIMS and the LC/MS approaches give comparable and successful discrimination of all exposure groups. More interesting, less intra-group dispersion is observed for DIMS data, enabling better group separation. Such observation

suggests that less technical drifts may occur in the high throughput DIMS approach compared to LC/MS. Importantly, a component characteristic of the control group despite heterogeneity among individuals could be provided by IC-DA for both data sets. Such characterization may be useful for large-scale epidemiological studies in which assignment of control subjects is required for biological interpretation or clinical assessment.

3.2. Detection and characterization of discriminant metabolites

The most relevant variables involved in the group discrimination of the five exposure types were extracted to be further identified. However, this step is not so easy when considering several exposure groups. It is less straightforward to highlight the contribution of a given variable to one (or more) specific exposure group(s) in our case, compared to classical studies which tend to find the difference between control and an unhealthy group [9,14,15]. Therefore, different computational tools were used to improve the selection of the most discriminant ions for every specific exposure group (see experimental section).

First, from the Q–Q plot only discriminant variables characterized by high standard deviations were extracted. 25 variables and 134 variables were selected from the DIMS and LC/MS data, respectively, with only four common variables among them. After concatenating very close m/z variables, the number of highly informative discriminant variables decreased to 17 and 115 for DIMS and LC/MS, respectively, and to 16 and 56 after application of a permutation procedure (see experimental section).

In a second step, box-whisker plots were used to show the ion abundance changes among all groups. The example of two discriminant variables at m/z 132.0763 and 138.0546

selected from DIMS data analysis is illustrated in **Fig. 3**. The response of m/z 132.0763 ions is enhanced for samples coming from farmers exposed to captan (**Fig. 3a**), suggesting a high contribution of these species to reveal captan exposure. For ions at m/z 138.0546, intensity change between groups is less clear **(Fig. 3b**). Their response is relatively low for the captan exposure group whereas a slightly higher response is obtained in the other exposure groups, *i.e.* those exposed to isoproturon, chlorothalonil and cyprodinyl. In this latter case, it is difficult to affirm the significant variation of peak intensity between different groups. The use of Student's t-test confirmed that 15 variables among the 16 previously selected were significant for the DIMS data (see experimental section), demonstrating the robustness of the DIMS approach (**Table 1**). In contrast, the use of the t-test decreased the number of significant variables from 56 to 29 for the LC/MS data (**Table S-1**). This indicates that these 27 removed variables are not related to biological perturbations but that their intensity was probably affected by technical drifts occurred over a long period of analysis.

Finally, correlation coefficients were calculated for each discriminant variable to help in metabolite annotation. The putatively annotated discriminant variables (in bold) and the correlated ions detected in the DIMS and LC/MS experiments are reported in **Table 1** and **Table S-1**, respectively. Among the annotated discriminant metabolites, several di or tri-peptides (*e.g.*, leucyl-proline), as well as amino-acids (*e.g.*, tryptophan) and amino acid metabolism intermediates (*e.g.*, creatine, creatinine) were selected from both DIMS and/or LC/MS data (**Table 1** and **Table S-1**). These di- and tri-peptides have been described as breakdown products of protein digestion or protein catabolism. However, their role in physiological function is not well known. Most of them have been reported

as short-lived intermediates formed during proteolysis (HMDB, *http://www.hmdb.ca*). Nicotinuric acid, kynurenic acid and xanthurenic acid ions were determined as discriminant variables from LC/MS data (see the **Table S-1**). They display a decreased intensity for subjects exposed to captan. Kynurenic acid has already been described as a metabolite structurally related to xanthurenic acid [30]. Additionally, high level of xanthurenic acid has been reported to characterize a metabolic perturbation, for example, as a putative inducer of malaria development in mosquitoes [30]. In our study, the detection of this metabolite may reflect metabolic disruption for subjects exposed to captan.

Alternately, acylglycine species (furoylglycine, nicotinuric acid, hippuric acid), and carnitine or acylcarnitine derivatives (dehydroxycarnitine, acetyl-carnitine, keto-decanoylcarnitine, dodecanedioylcarnitine, decadienylcarnitine) were proposed for the annotation of some discriminant variables (**Table 1** and **Table S-1**). These metabolites have been described as indicators of disturbed metabolic pathways [31–34]. The unusual acylcarnitine excretion levels have been reported to be related to diseases such as obesity, type 2 diabetes, cardiovascular disease, encephalopathy and carcinoma [32,35]. In our study, the discriminant ions at m/z 204.1230 could be annotated as acetylcarnitine based on their accurate m/z value (*i.e.* $C_9H_{18}NO_4$ formulae with 0.5 ppm). However, by comparing with the CID mass spectrum of the authentic standard compound, the seemingly differences in the fragment ion abundances suggest that the m/z 204.1230 ions could correspond to an isomer of acetylcarnitine [36] (**Fig. S-6** of the Supplementary materials).

Another example concerns ions correlated to the discriminant ions at m/z 76.0753. The **Fig. 4** displays a reconstituted DIMS mass spectrum of a urine sample collected on a subject exposed to chlorothalonil. The m/z 76.0753 ions were putatively annotated as

$[M + H]^+$ ions of trimethylamine N-oxide ($C_3H_{10}NO$, 5.3 ppm), an oxidation product of the trimethylamine. This compound has been described as a marker of oxidative stress. Its high concentration has been reported in transplant patients, reflecting the effects of kidney diseases [37]. These m/z 76.0753 ions appear to be related to ions at m/z 98.0573, 136.1077, 151.1438, 152.1471 and 189.1345. The presence of ions at m/z 98.0573, 151.1438 and 152.1471 reinforced the assignment of elemental composition of the m/z 76.0753 ions ($C_3H_{10}NO$, 5.3 ppm) since they could correspond to different species of trimethylamine N-oxide such as $[M + Na]^+$ (C_3H_9NONa, 3 ppm), $[2M + H]^+$ ($C_6H_{19}N_2O_2$, 3 ppm) and ^{13}C isotope peaks of the $[2M + H]^+$ ($^{13}C_1{}^{12}C_5H_{19}N_2O_2$, 3 ppm) ions, respectively. Additionally, the correlated ions at m/z 136.1077 and 189.1345 could be annotated as $[M + NH_4]^+$ ions of urea, N-hydroxy-N-propyl ($C_4H_{10}N_2O_2$, 3 ppm) and $[M + H]^+$ ($C_7H_{17}N_4O_2$, 1 ppm) ions of homo-L-arginine or their isomers, respectively. A biological connection between these three compounds has already been reported by He et al. [38].

4. Conclusion

Discrimination of individuals exposed to the five pesticides was successfully obtained with both DIMS and LC/MS data. This result demonstrates the applicability of DI-HRMS approach for metabolomic investigations involving several groups of subjects. More importantly, the very short data acquisition time makes the DIMS a valuable approach for performing high throughput metabolomic fingerprinting, in particular for large scale epidemiological investigations which aim to find associations between exposure to toxicants and their effects on human health.

Besides, our approach enabled the detection of some metabolites already described in the literature (*i.e.* urea, creatinine, carnitine, acylcarnitine, acetylcarnitine, dehydroxycarnitine, keto-decanoylcarnitine, dodecanedioylcarnitine) as indicators of toxic exposure. Pesticide exposure has been reported to cause significant DNA damage [20], possible effects on fetuses in pregnant women [19] and cardiac dysfunction [39]. Such an exposure could impact metabolism pathways resulting in changes of metabolite concentrations [19,34,40–42]. Further studies are needed to provide more accurate toxicological evaluation and precise identification as well as biological interpretation of biomarkers specifically related to pesticide exposure.

Electronic supplementary information

Supplementary data related to this article can be found: LC/MS experimental procedure and additional results illustrated by Figures S-1 to S-6 and table S-1.

Conflicts of interest

The authors declare no conflict of interest.

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Table and Figure captions

Table 1 Annotation of the most discriminant m/z values and their correlated ions from DIMS dataset.

Fig. 1 Discrimination of control subjects (**a**) and exposure classes: captan (**b**), chlorothalonil (**c**), cyprodinyl (**d**), and isoproturon (**e**). Results obtained from DIMS data

processing without the contribution of group information, *i.e.*, based only on the extracted MS source signals. Note that discrimination of epoxiconazol-exposed group is visible in another dimension (**b, c and e**).

Fig. 2 3D-plots obtained from DIMS (**a**) and LC/MS (**b**) data processing without the known group memberships.

Fig. 3 Box-whisker plots showing intensity changes for two discriminant variables, m/z 132.0763 and 138.0546 (from DIMS dataset) between control and all exposure groups. The box represents the first and third quartiles, the bold horizontal lines represent the median intensity. The whiskers correspond to the 1.5 times the interquartile range, and the asterisk represent the outlier.

Fig. 4 Zoomed mass spectrum (in the m/z 60-200 range) reconstituted from correlation coefficients calculated from DIMS dataset of a urine sample collected on a subject exposed to chlorothalonil. Color coding of the signal points is based on their correlation with the discriminant variables at m/z 76.075. The peaks in red were strongly correlated.

Table 1.

Observed peak (m/z)	Proposed ion elemental composition	Putative ion annotation [a]		** Isoprotunron	Captan	Chlorothalonil	Cyprodinil	Epoxiconazol
		Proposed species	Proposed compound name					
76.0753	$C_3H_{10}NO$	$[M+H]^+$	Trimethylamine N-oxide	-	-	↗	-	↘
98.0573	C_3H_9NONa	$[M+Na]^+$		↘	-	-	↘	↘
151.1438	$C_6H_{19}N_2O_2$	$[2M+H]^+$		-	-	↗	↘	↘
152.1471 [b]	$^{12}C_5{}^{13}CH_{19}N_2O_2$	$[2M+H,^{13}C]^+$		↘	-	-	↘	↘
136.1077	$C_4H_{10}N_2O_2NH_4$	$[M+NH_4]^+$	Urea, N-hydroxy-N-propyl					
189.1345	$C_7H_{17}N_4O_2$	$[M+H]^+$	Homo-arginine	-	↘	-	-	-
132.0763	$C_4H_{10}N_3O_2$	$[M+H]^+$	Creatine	↘	↗	↘	↘	↘
110.0288				↗	↘	-	↗	-
138.0547	$C_7H_8NO_2$	$[M+H]^+$	Trigonelline	↗	↘	-	↗	-
140.0452	$C_5H_6N_3O_2$	$[M+H]^+$	Hydroxypyrazinamide					
144.1016	$C_7H_{14}NO_2$	$[M+H]^+$	Proline betaine	-	-	-	↗	↘
145.1049 [b]	$^{12}C_6{}^{13}CH_{14}NO_2$	$[M+H,^{13}C]^+$						
229.1545	$C_{11}H_{21}N_2O_3$	$[M+H]^+$	Leucyl-Proline	-	-	-	↗	-
230.1578 [b]	$^{12}C_{10}{}^{13}CH_{21}N_2O_3$	$[M+H, ^{13}C]^+$						
241.1543	$C_{12}H_{21}N_2O_3$	$[M+H]^+$	Pirbuterol					
115.0626 [b]	$C_4H_8{}^{14}N_2{}^{15}N_1O$	$[(M+H), ^{15}N]^+$	Creatinine					
115.0689 [b]	$^{12}C_3{}^{13}C\ H_8N_3O$	$[(M+H),^{13}C]^+$						
227.125	$C_8H_{15}N_6O_2$	$[2M+H]^+$						
228.1280 [b]	$^{12}C_7{}^{13}CH_{15}N_6O_2$	$[2M+H,^{13}C]^+$		-	↘	-	-	-
249.1069	$C_8H_{14}N_6O_2Na$	$[2M+Na]^+$						
362.1659	$C_{12}H_{21}N_9O_3Na$	$[3M+Na]^+$						
162.1122	$C_7H_{16}NO_3$	$[M+H]^+$	Carnitine	↘	-	↘	↘	↗

163.1157 [(b)]	$^{12}C_6{}^{13}CH_{16}NO_3$	$[M+H, {}^{13}C]^+$							
146.1171	$C_7H_{16}NO_2$	$[M+H]^+$	Dehydroxycarnitine						
204.1229	$C_9H_{18}NO_4$	$[M+H]^+$	Acetylcarnitine	-	↗	-	↗	-
118.0605	$C_3H_8N_3O_2$	$[M+H]^+$	Guanidoacetic acid	↘	-	-	↘	-
257.1604	$C_{11}H_{22}N_4O_4$	$[M+H-H_2O]^+$	Glutaminyl-Lysine	-	↘	↗	-	-

⁂ Changes between each exposed group and their control one: -, no change; ↗, increase; ↘, decrease.

Horizontal lines separate each set of correlated ions; bold m/z values correspond to the selected discriminant variables; the non-bold m/z values are correlated variables with the first one (in bold). [(a)] Note that only one identity was assigned for each variable among the different isomers proposed from DB queries. [(b)] Proposed ^{13}C and ^{15}N isotopic peaks. Correlated ions without DB hit were eliminated from the list.

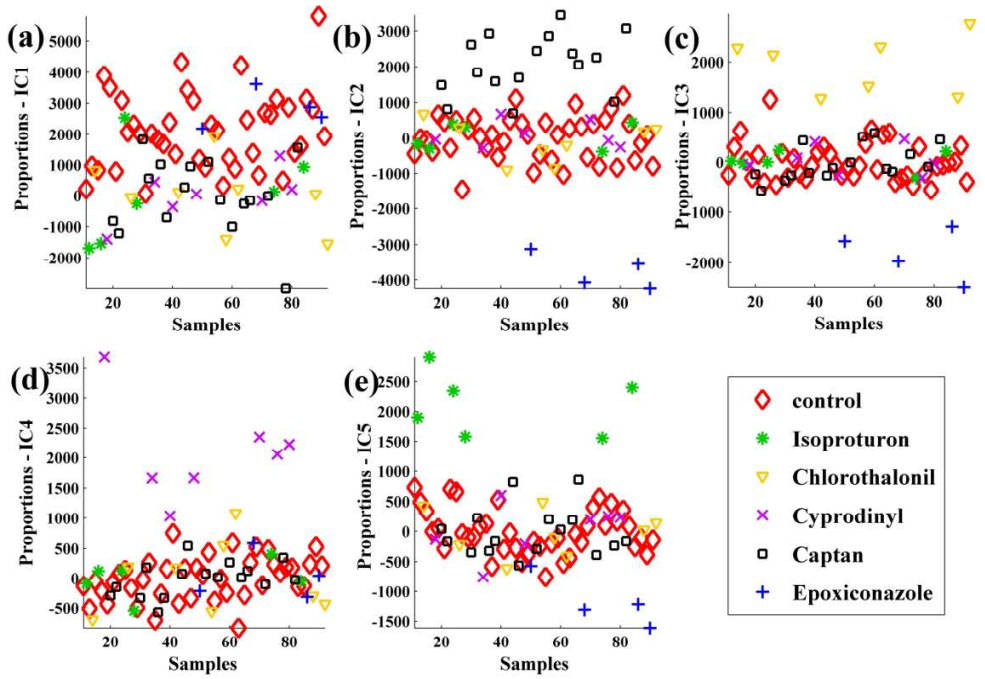
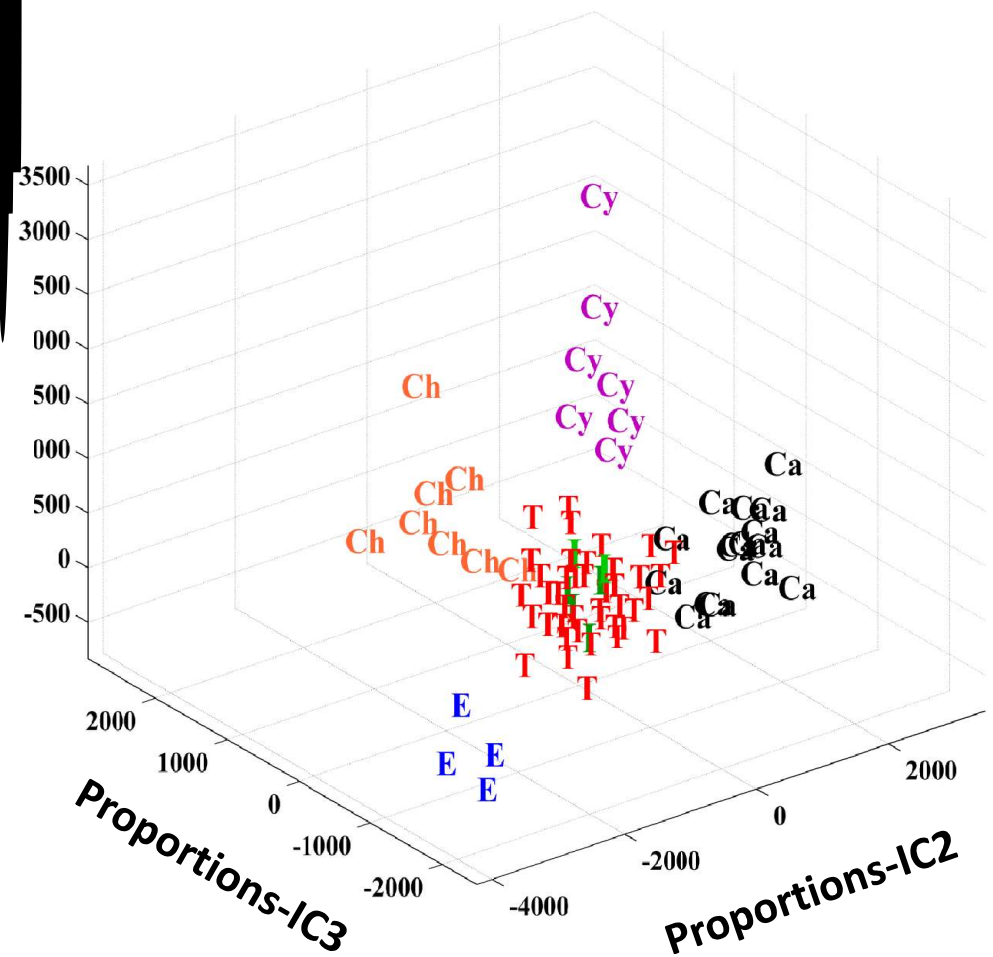
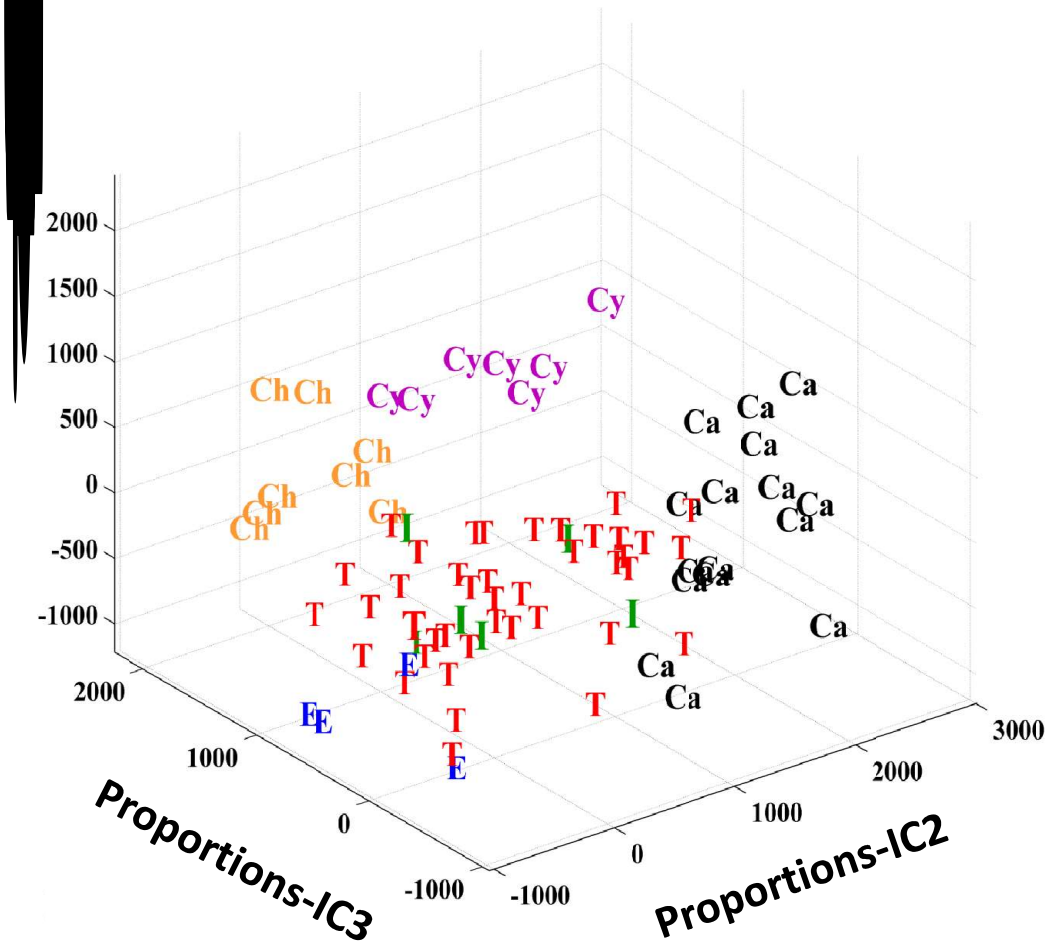


Fig. 1 Discrimination of control subjects (a) and exposure classes: captan (b), chlorothalonil (c), cyprodinyl (d), and isoproturon (e). Results obtained from DIMS data processing without the contribution of group information, i.e., based only on the extracted MS source signals. Note that discrimination of epoxiconazol-exposed group is visible in another dimension (b, c and e).

212x150mm (300 x 300 DPI)

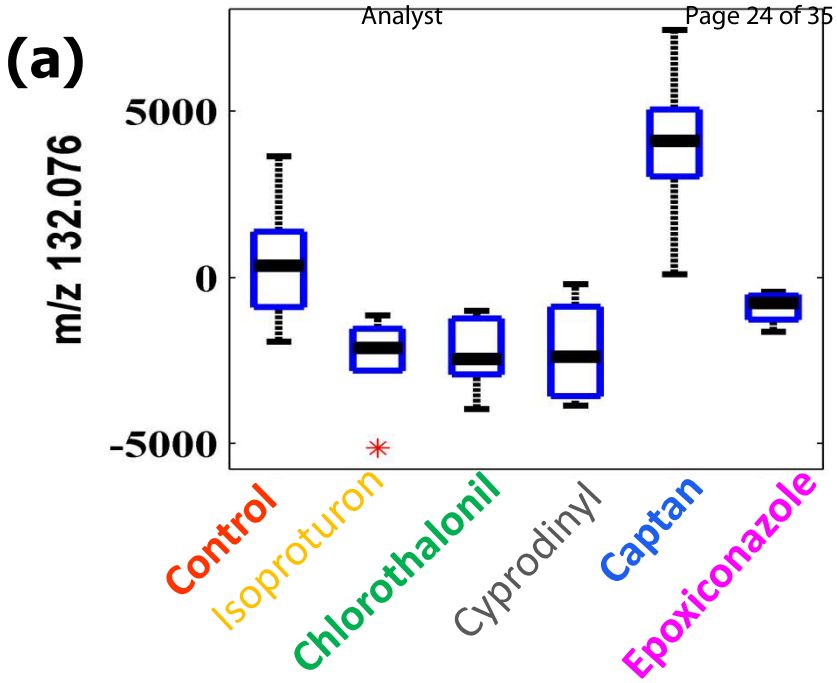


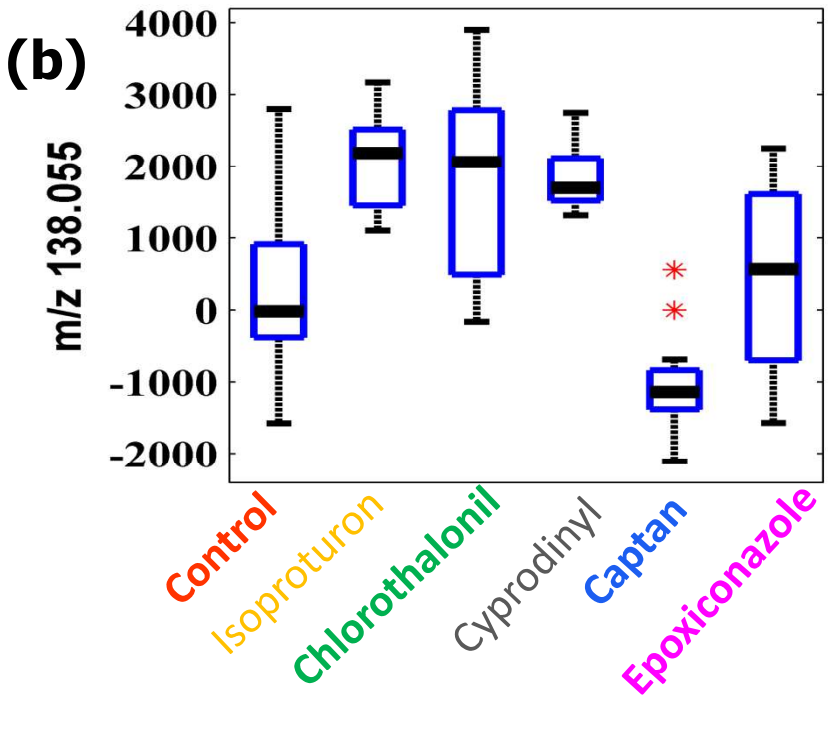
(a)

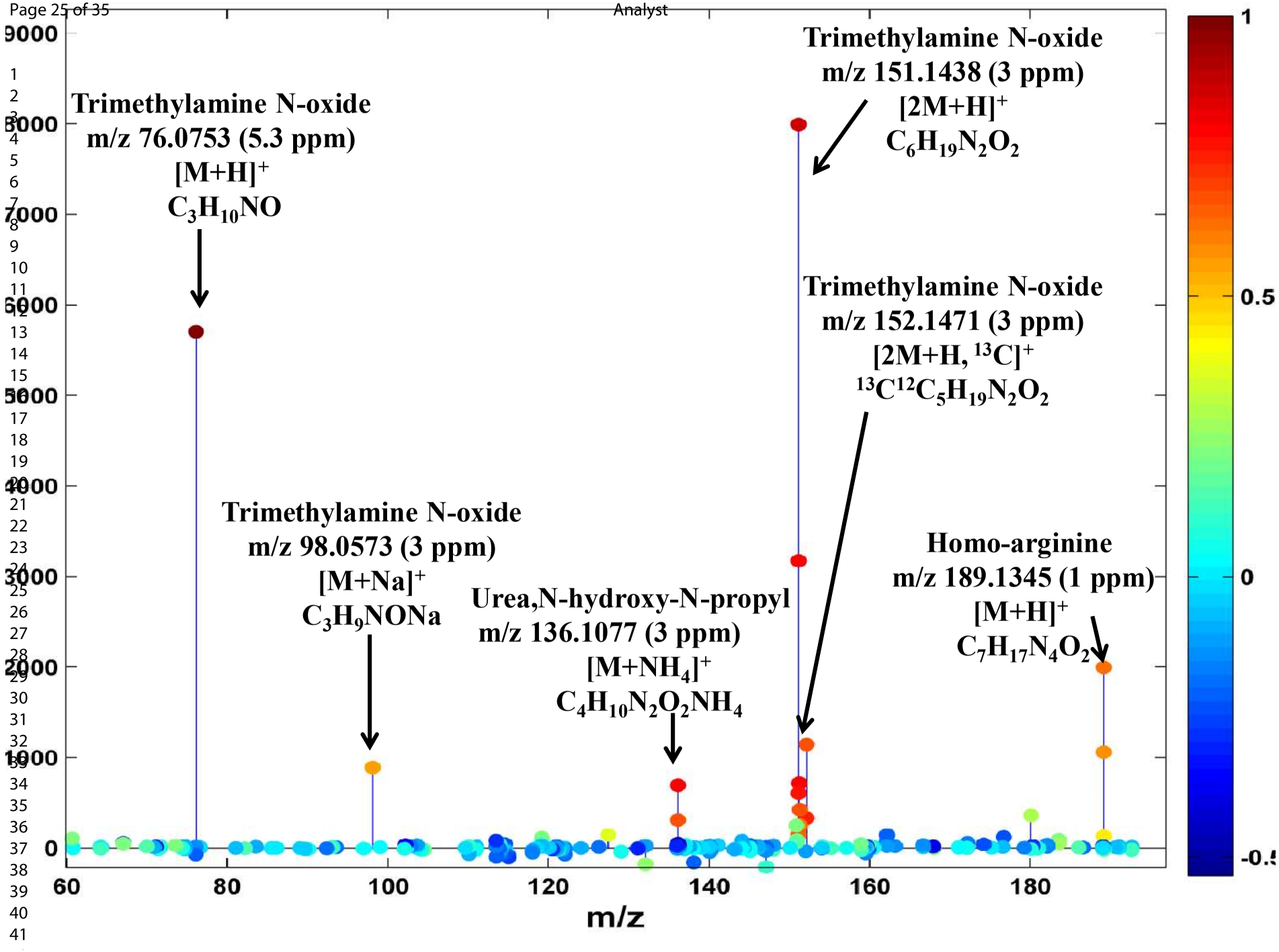


(b)

Ca captan Ch chlorothalonil Cy cyprodinyl E epoxiconazol I isoproturon T controls


(a)
m/z 132.076
5000
0
-5000
Control
Isoproturon
Chlorothalonil
Cyprodinyl
Captan
Epoxiconazole


(b)
m/z 138.055
4000
3000
2000
1000
0
-1000
-2000
Control
Isoproturon
Chlorothalonil
Cyprodinyl
Captan
Epoxiconazole


Trimethylamine N-oxide
m/z 76.0753 (5.3 ppm)
[M+H]+
C3H10NO
Trimethylamine N-oxide
m/z 151.1438 (3 ppm)
[2M+H]+
C6H19N2O2
Trimethylamine N-oxide
m/z 152.1471 (3 ppm)
[2M+H, 13C]+
13C12C5H19N2O2
Trimethylamine N-oxide
m/z 98.0573 (3 ppm)
[M+Na]+
C3H9NONa
Urea,N-hydroxy-N-propyl
m/z 136.1077 (3 ppm)
[M+NH4]+
C4H10N2O2NH4
Homo-arginine
m/z 189.1345 (1 ppm)
[M+H]+
C7H17N4O2
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
60
80
100
120
140
160
180
m/z
1
0.5
0
-0.5

Supplementary Material

Evaluation of direct introduction-high resolution mass spectrometry (DI-HRMS) based metabolomics approach for the discrimination of several exposure groups to pesticides: comparison with liquid chromatography high resolution mass spectrometry (LC/HRMS)

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Supplementary material section contains LC/MS and MS/MS experimental conditions. Additional results obtained from both DIMS and LC/MS data are presented in Fig. S-1 to S-5. Table S-1 showed the most discriminant variables and their correlated ions putatively annotated from LC/MS data.

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LC/MS experimental procedure

1. Mass spectrometry detection

LC/MS experiments were acquired in the m/z 100-1000 range with a cycle time of 14 min (including column re-equilibration) using a mass resolving power of 100,000 FWHM (Full width at half maximum) at m/z 400. The following ESI conditions were applied: electrospray voltage was set at -3 kV, capillary voltage at 3 V, capillary temperature at 275 ˚C and the tube lens offset at 80 V, sheath gas flow (nitrogen) and auxiliary gas were fixed at 66 and 30 arbitrary units (a.u.), respectively.

The chromatographic separation was performed using a C_{18} NucleodurHTec column (2 × 100 mm, 3 µm, Macherey-Nagel, Eurl, Hoerdt, France) and a high performance liquid chromatography system, Surveyor LC (Thermo Fisher Scientific). 10 µL of sample diluted 10 fold in a mixture of acetonitrile/water (1:1, v/v) were injected at a flow rate of 200 µL/min. The mobile phase consisted of water plus 0.1 % formic acid (A) and acetonitrile plus 0.1% formic acid (B). The gradient started with 2% (B) for 1 min, and increased to 100% (B) in 7 min, then was maintained at 100% (B) during 2 min.

2. Data pre-processing

Raw LC/MS data were converted from profile into centroid mzXML format using MS Convert (http://proteowizard.sourceforge.net/tools.shtml). XCMS based on a program written in R (*R version 3.4.0, The R Foundation for Statistical Computing, Vienna 2017, https://www.r-project.org*) was used to pre-process generated data. Four basic steps corresponding to the peak picking, the peak grouping, the retention time correction, and a second peak grouping were performed. The *centWave* method was used for peak extraction, followed by the correction of retention time drift using a non-linear LOESS alignment method (*Smith C. A. et al., XCMS: Processing Mass Spectrometry Data for Metabolite Profiling Using Nonlinear Peak Alignment, Matching, and Identification.2006, Anal Chem 78:779–787*). Finally, the fill peaks method was applied to find peak intensity in the raw data to correct missing values. The final number of extracted variables from the 82 samples was 2106.

MS/MS experiments

Tandem mass spectrometry (MS/MS) experiments were performed on the monoisotopic peaks of some selected metabolites using CID (collision-induced dissociation) and/or HCD (higher collision dissociation) conditions. The following parameters were used: isolation width of precursor ions of 1 to 2 u, activation time of 30 ms, and normalized collision energy of about 20 % and 38 % (arbitrary units) for CID and HCD, respectively. The precursor ions were selected, fragmented in the linear ion trap (LTQ) device and final high-resolution detection with a mass resolving power of 7500 (FWHM) for m/z 400 was performed.

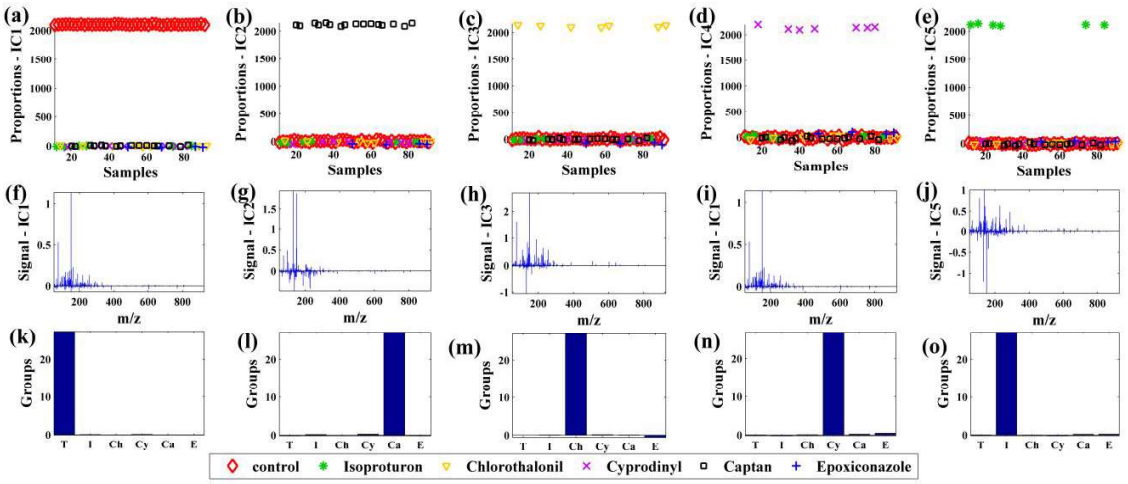

Fig.S.1 Results obtained from the DIMS data processing with known group memberships. Discrimination of control subjects (**a**) and the four exposure classes: captan (**b**), chlorothalonil (**c**), cyprodinyl (**d**) and isoproturon (**e**). The corresponding extracted source signals show the m/z values which most contribute to group discrimination (**f**, **g**, **h**, **i** and **j**). The peak intensity represents the ion contribution to the corresponding IC. The part of the source signals belonging to the group membership matrix show which group(s) contribute(s) the most to build the IC when compared to the influence of the others (**k**, **l**, **m**, **n** and **o**). Note that no discrimination is observed for epoxiconazol-exposed subjects.

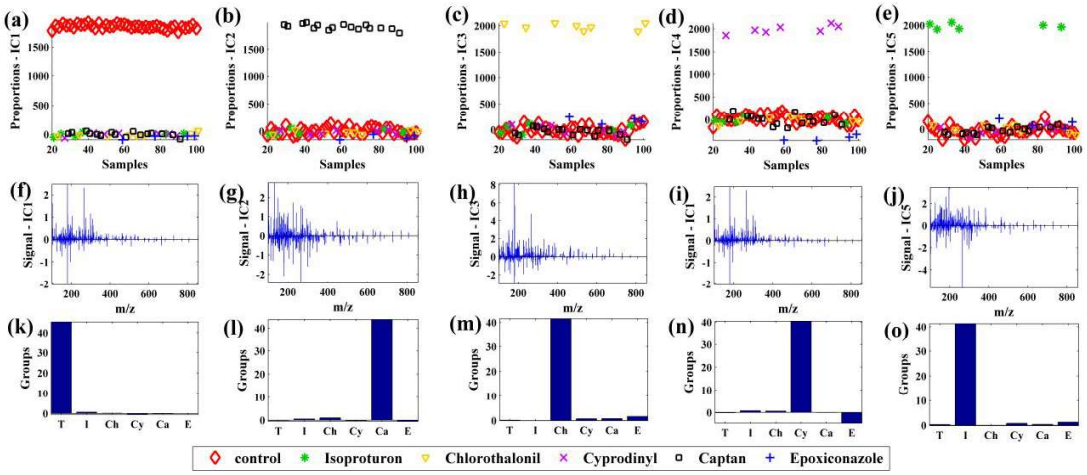

Fig.S.2 Results obtained from LC/MS data processing with known group memberships. Discrimination of control subjects (**a**) and all exposure classes: captan (**b**), chlorothalonil (**c**), cyprodinyl and epoxiconazol (**d**) and isoproturon (**e**). The corresponding extracted source signals show the m/z values which most contribute to group discrimination (**f**, **g**, **h**, **i** and **j** plots). The peak intensity represents the ion contribution to the corresponding IC. The part of the source signals belonging to the group membership matrix show which group(s) contribute(s) the most to build the IC when compared to the influence of the others (**k**, **l**, **m**, **n** and **o**).

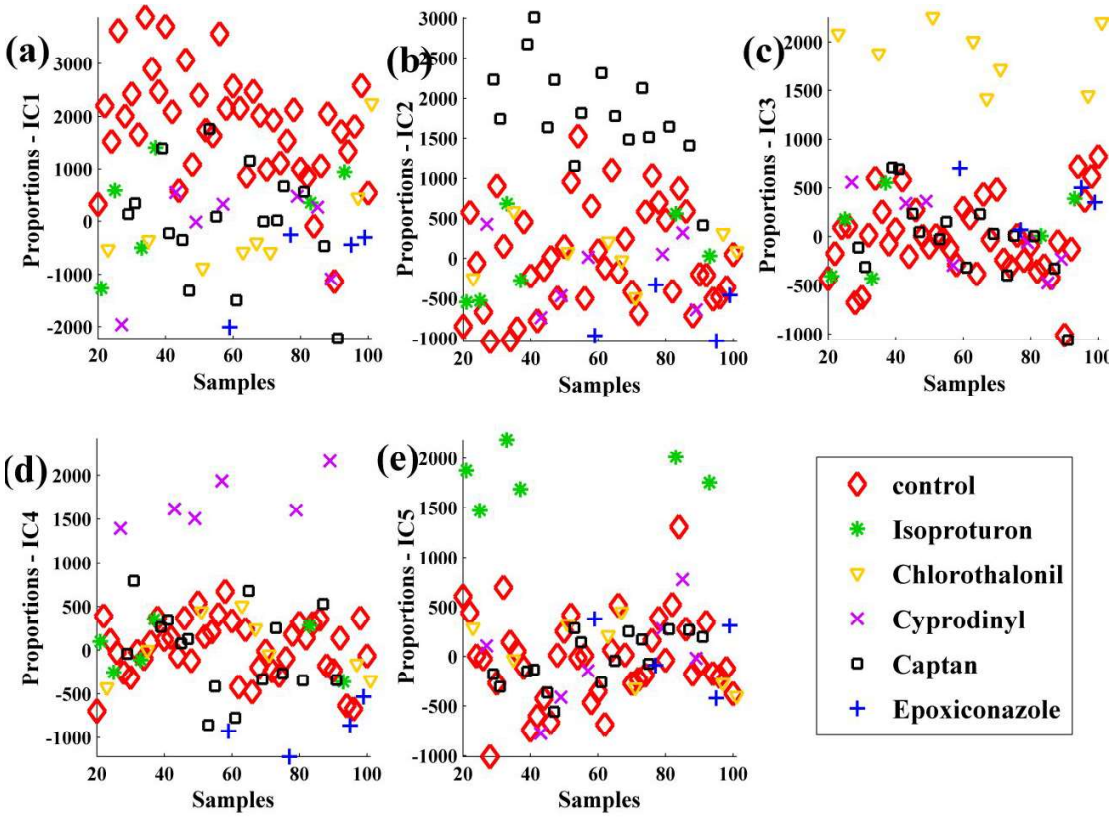

Fig.S.3 Results from LC/MS data processing without the contribution of group information, i.e., based on the only extracted MS source signals. A separation is still observed for captan, chlorothalonil, cyprodinil and isoproturon exposure groups (**b-e** respectively). A discrimination of control subjects (**a**) is less apparent and no discrimination of epoxiconazol exposure group is obtained.

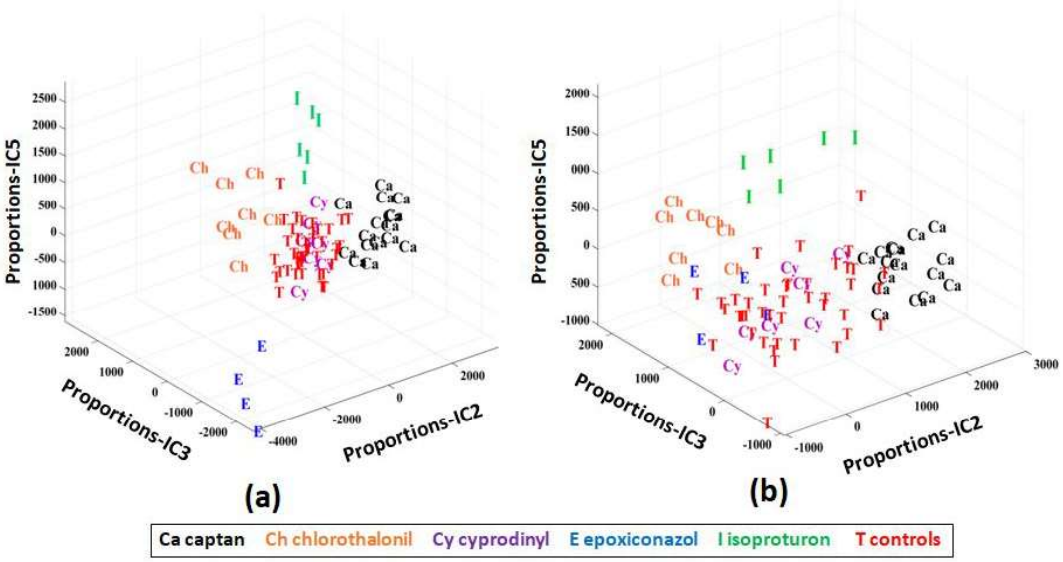

Fig.S.4 3D-plot from DIMS (**a**) and LC/MS (**b**) data processing without the contribution of group information. Discrimination of different exposure groups is observed, especially, separation of individuals exposed to isoproturon according to the proportions of IC-5. Cyprodinyl exposure group was discriminated according to another dimension as observed in **Fig.2**.

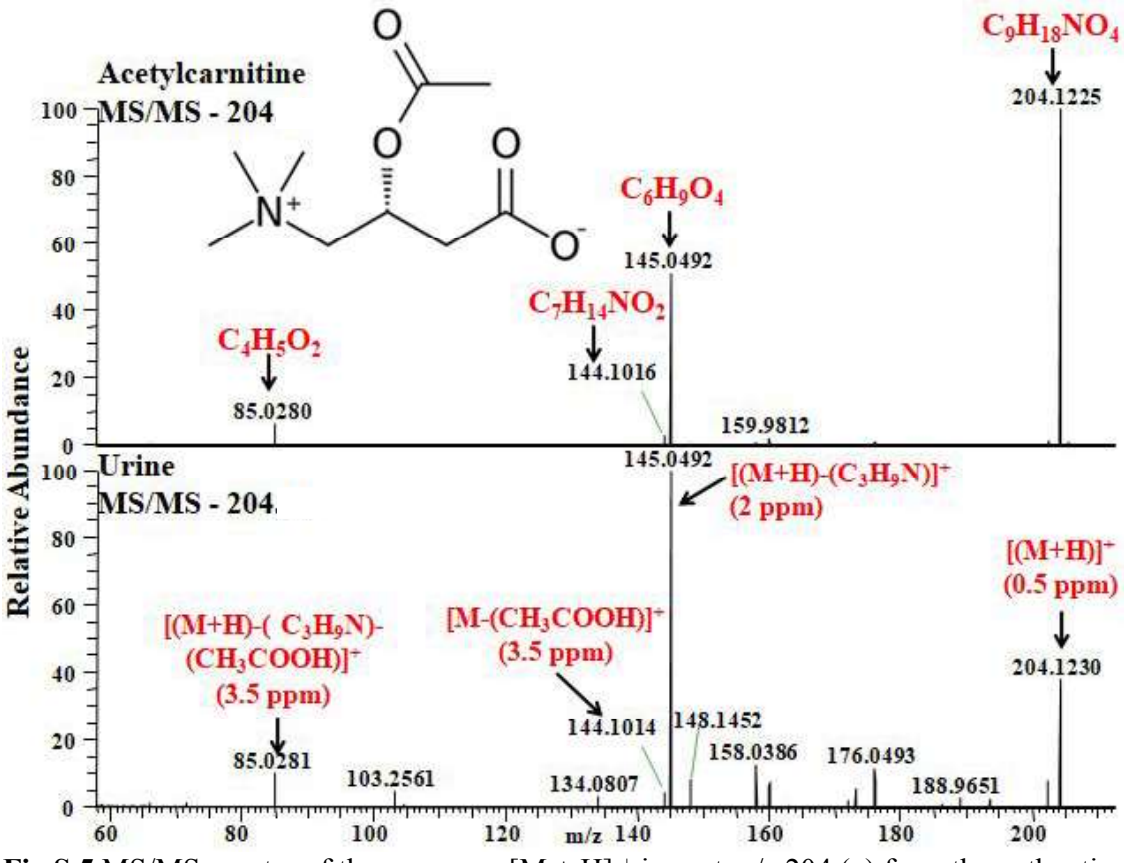

Fig.S.5 MS/MS spectra of the precursor $[M + H]^+$ ions at m/z 204 (**a**) from the authentic standard acetylcarnitine and (**b**) from a urine sample.

Table S.1 The most discriminant m/z values extracted from LC/MS data

Observed peak(m/z)	Proposed ion elemental composition	Putative ion annotation [a]		Isoprotunron	Captan	Chlorothalonil	Cyprodinil	Epoxiconazol
		Proposed species	Proposed compound name					
102.0524; 736	$C_2H_9NO_2Na$	$[M+Na]^+$	N-Hydroxy-N-methylmethanamine-water	-	-	-	-	↘
102.0823; 771				-	-	↘	-	↘
102.1069; 549								
271.1515;776	$C_{10}H_{19}N_6O_3$ $C_{10}H_{19}N_6O_3$	$[M+H-H_2O]^+$ $[M+NH_4]^+$	GlyGlyArg 3-[2-(6-Amino-9H-purin-9-yl)ethoxy]propane-1,2-diol					
250.3262;798								
381.2792;795	$C_{26}H_{37}O_2$	$[M+H]^+$	Benzoic acid 2-[(4-dodecylphenyl)methyl]					
102.1271; 750						↗*		
102.1402; 694				-	↗	-	-	-
102.1406;805				-	↗	-	-	-
102.1069;549								
102.1481;760				-	↗	-	↗	-
102.1054;518								
102.1483; 723				-	-	-	-	↘
114.0595;591				-	-	-	↗	-
114.0896; 589	$C_4H_{10}N_4$	$[M+NH_4]^+$	Methyl-triazin-ium	-	↗	-	↗	-
121.1058;103				-	↘	-	-	-
136.0478; 63	$C_4H_7N_3ONa$	$[M+Na]^+$	Creatinine	-	-	↘	-	-
141.1131;33	$C_6H_{13}N_4$	$[M+H]^+$	1-(2-Methylbutan-2-yl)-1H-tetrazole	-	-	-	-	↘
150.1447;75				-	↘	-	↘	↗
118.5554;94								
118.6349;74								
118.6495;306								
150.1702;77								
150.1998;74								
195.9931;306								
196.0355; 53	$C_7H_6N_3O_4$	$[M+H]^+$	1,2,4-Oxadiazole, 5-methyl-3-(5-nitro-2-furanyl)					
196.0366;136	$C_{10}H_7NO_2Na$ $C_9H_8O_5$	$[M+Na]^+$ $[M+H]^+$	Quinaldic acid 3-Carboxy-4-(methoxycarbonyl)phenolate					
384.2627; 74	$C_{23}H_{34}N_3O_2$ $C_{18}H_{34}N_5O_4$	$[M+H]^+$ $[M+H-H_2O]^+$	N-Dodecyl-2-(1H-imidazole-carbonyl)benzamide 2AlaAla Lys Leu-	-	↘	-	-	-
170.0410; 50	$C_8H_6F_2NO$	$[M+H]^+$	3,5-Difluoro-4-(hydroxymethyl)benzonitrile	-	-	↗	-	-
103.5474;53								
129.0141; 50	$C_4H_{10}SK$ C_9H_2F	$[M+K]^+$ $[M+H-2H_2O]^+$	1-Butanethiol Fluorochromone					
153.0326; 50	$C_3H_9N_2O_3S$	$[M+H]^+$	(Methylamino)(methylimino)met					

			hanesulphonic acid					
189.0468; 50	$C_8H_{10}N_2S_1Na$	$[M+Na]^+$	Ethionamide					
170.0447; 206	$C_7H_8NO_4$	$[M+H]^+$	Furoylglycine			↗*		
180.0675; 484	$C_7H_7FN_5$	$[M+H]^+$	4-Fluoro-3-(1H-tetrazol-1-yl)aniline	↗	-	↗	-	-
180.1005; 480	$C_8H_{15}NO_2Na$	$[M+Na]^+$	Homostachydrine	-	↘	-	↗	-
181.0608; 447	$C_8H_9N_2O_3$	$[M+H]^+$	Nicotinuric acid	-	↘	-	-	-
190.0498; 480	$C_{10}H_8NO_3$	$[M+H]^+$	Kynurenic acid					
206.0448; 463	$C_{10}H_8NO_4$	$[M+H]^+$	Xanthurenic acid					
181.0687;484[(b)]	$C_8{}^{13}CH_{10}NO_3$	$[M+H, {}^{13}C]^+$	Hippuric acid			↘*		
105.0154;483[(b)]	C_7H_5O	$[M+H-C_2H_5NO_2]^+$						
179.0188;480	$C_6H_{10}O_2S_2$	$[M+H]^+$	Bissulfine					
181.1212; 480	$C_9H_{15}N_3O$	$[M+H-2H_2O]^+$	Maleic hydrazide choline salt	-	-	↗	-	-
205.0972; 267	$C_{11}H_{13}N_2O_2$	$[M+H]^+$	Tryptophan	-	↘	-	-	-
242.9254; 60				-	-	↘	-	-
174.938; 60								
310.9128;60								
446.8873;60								
265.1220; 477	$C_{18}H_{17}O_2$	$[M+H]^+$	Cinnamylcinnamate	-	↘	-	-	↘
274.122;390	$C_{19}H_{16}NO$	$[M+H]^+$	Methoxyphenylcarbazole	-	-	-	-	↘
	$C_{11}H_{20}N_3O_3S$	$[M+H-H_2O]^+$	CysLeuGly					
114.0497;463								
114.0569;459	$C_4H_{12}PNa$	$[M+Na]^+$	Phosphonium, tetramethyl					
299.1828; 750	$C_{12}H_{23}N_6O_3$	$[M+H-H_2O]^+$	Ala AlaArg	-	↘	-	-	-
310.2011; 497	$C_{17}H_{28}NO_4$	$[M+H]^+$	Decadienylcarnitine			↘*		
311.2044; 497[(b)]	$^{12}C_{16}{}^{13}CH_{28}NO_4$	$[M+H,{}^{13}C]^+$						
304.4917;497								
286.1716;528	$C_{18}H_{25}NP$	$[M+H]^+$	Ethanamine, 2-(diphenylphosphino)-N,N-diethyl					
326.0869; 433	$C_{14}H_{16}NO_8$	$[M+H]^+$	Dihyroxy-1H-indole glucuronide I	-	↘	-	-	-
348.0687; 433	$C_{14}H_{15}NO_8Na$	$[M+Na]^+$						
464.1909;528	$C_{23}H_{30}NO_9$	$[M+H]^+$	Dihydroisomorphine-glucuronide					
330.2273; 487	$C_{17}H_{32}NO_5$	$[M+H]^+$	Keto-decanoylcarnitine	-	-	-	↘	-
331.2306; 487[(b)]	$^{12}C_{16}{}^{13}CH_{32}NO_5$	$[M+H,{}^{13}C]^+$						
374.2533; 490	$C_{19}H_{36}NO_6$	$[M+H]^+$	Dodecanedioylcarnitine					
229.1433; 514	$C_{12}H_{21}O_4$	$[M+H]^+$	Traumatic acid					
269.1494; 504	$C_{11}H_{25}N_3O_2$	$[M+K]^+$	Diacetylspermidine					
	$C_{13}H_{21}N_2O_4$	$[M+H]^+$	Dihydroxymelphalan					
356.2429; 507	$C_{19}H_{33}NO_5$	$[M+H]^+$	Derivative of carnitine					

‡ Changes between each exposed group and their control one: -, no change; ↗, increase; ↘, decrease; change between all exposed groups and all control: ↗*, increase; ↘*, decrease. Horizontal lines separate each set of correlated ions; bold m/z value correspond to the most discriminant variables; the non-bold m/z values are correlated variables with the first one (in bold). [(a)] Note that only one identity was assigned for each variable among the different isomers proposed from DB queries.[(b)] Proposed ion fragment composition or ^{13}C isotopic peak. Correlated ions without DB hit were eliminated. Some candidates considered unreliable were rejected in the absence of isotopic peaks on the mass spectrum (e.g., absence of the ^{37}Cl isotopic peak in the mass spectrum since its expected abundance should correspond to 30% and 60 % for one and two chlorine atoms, respectively, compared to the monoisotopic peak).