

LC-MS/MS Based Metabolomics to Identify Biomarkers Unique to *Laetiporus sulphureus*

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Abstract: LC/MS/MS technique, employing QTOF mass analyzer, was used for comparative metabolomic fingerprinting of seven edible mushroom varieties (*P.ostreatus*, *L.edodes*, *L.sulphureus*, *A.campestris*, *T.clypeatus*, *T.microcarpus* and *T.letestui*). The aim was to identify biomarkers unique to *L.sulphureus* which might be responsible for the pharmacological claim of the mushroom by the Kaffa people in Ethiopia. As an outcome of the data mining and pre-treatment step using MarkerviewTM software, positive and negative ionization data matrices of 71,083 and 54,856 peaks, respectively, were obtained. Regardless of the ionization mode, the principal component analysis (PCA) of the data set representing the seven edible mushrooms each in triplicate revealed a unique separate clusters for *L.sulphureus*, documenting differences in LC-MS profiles associated with the sample. Based on plot profile, only 14 and 27 peaks representing monoisotopic ions unique to *L.sulphureus* at the positive and negative ionization mode respectively were obtained. All the pre-selected biomarkers were searched from METLIN metabolite database, but only one peak at 13.41 min with m/z of 471.3468 and 469.3348, positive and negative ionization, respectively were tentatively identified as 18 α -glycyrrhetic acid (commonly called Enoxolone). This metabolite was verified by comparing the retention time, MS and MS/MS data spectra of authentic standard and sample obtained from PeakviewTM software. Mass frontier software was used to generate possible fragmentation and rearrangement mechanisms of the parent ion. In conclusion, 18 α -glycyrrhetic acid might be one of the compounds responsible for the biological claim of the local people.

Keywords: Mushroom, LC-MS/MS, Metabolomics, PCA, Biomarkers, 18 α -Glycyrrhetic Acid

1. Introduction

Fungi have proved to be a fertile source of structurally diverse bioactive metabolites, which have yielded some of the most important products of the pharmaceutical industry. These include antibiotics (penicillin, cephalosporin), immunosuppressive agents (Cyclosporins, Rapamycin), cholesterol-lowering agents (Mevastatin, Pravastatin), psychotherapy (Lysergic Acid Diethylamide, psilocybin) are examples supporting today's great interest in new secondary metabolites from fungi (Cragg & Newman, 2005). Mushrooms, which are the sub class of fungi, have also been reported as therapeutic foods, useful in preventing diseases such as hypertension, hypercholesterolemia, atherosclerosis and cancer. These functional characteristics are mainly due to their chemical composition (Crisan &

Sands 1978; Kurasawa & Sugahara 1982; Manzi et al. 2001).

In Ethiopia, wild mushrooms are used as both food and medicine by various ethnic groups in the country. The habit of eating mushrooms differs from region to region and among the different ethnic groups of the same region. Interestingly the many tribes of southwest Ethiopia have a strong tradition of consuming mushrooms and are mycophilic. As a result, the ethnopharmacological knowledge accumulated and passed through generations about the medicinal value of the mushrooms were huge (Abate 1998). Kaffa zone is in southwest Ethiopia and it is the birthplace of coffee, hence the similarity in name Kaffa-Coffee. The Kaffa people use the fruiting body of

Laetiporus sulphureus to relieve stomach pain and to expel a woman's retained placenta following delivery (personal communication). However, no clinical studies have been conducted to validate the medicinal claims ascribed to this mushroom and no chemical compound (biomarker) unique to the mushroom has been identified as a possible agent responsible for the mushroom's metabolic effects.

The field of metabolomics, aiming at global analysis of numerous targeted or non-targeted low molecular compounds (metabolites) in a biological sample, has recently found its application in diverse research areas (Kaddurah-Daouk and Krishnan 2008; Wishart, 2008) including food quality and authenticity assessment (Cevallos-Cevallos *et al.* 2009); environmental & biological-stress studies (Rosenblum *et al.* 2005), biomarker discovery (Griffiths *et al.* 2010), functional genomics (Saito & Matsuda 2010) & integrative systems biology (Oliver *et al.* 1998).

Rapid growth of metabolomics has been enabled by substantial advances in analytical techniques such as mass spectrometry (MS) coupled to LC, GC, or CE and nuclear magnetic resonance (NMR), all techniques facilitating analysis of a wide range of metabolites with diverse physicochemical properties, occurring at different concentration levels (Dunn and Ellis 2005). There has been tremendous progress in mass spectrometry-based metabolomics recently, leaving researchers with a variety of choices for chromatographic separation, ionization, and mass spectrometric analysis. Mass spectrometer options include quadrupoles and ion traps which offer good sensitivity but limited resolving power (Glish & Burinsky 2008), or higher mass-resolution instruments such as time-of-flight (TOF) (Lacorte & Fernandez-Alba 2006), Fourier transform ion cyclotron resonance (FTICR) (Kelleher *et al.* 1999) or Orbitrap (Makarov *et al.* 2006). The mass spectrometer can also be arranged in a tandem configuration, such as a triple quadrupole mass spectrometer. Different types of analyzer can also be combined to form a hybrid mass spectrometer (Glish & Burinsky 2008), such as a quadrupole-TOF (QTOF) instrument or an ion trap-Orbitrap.

To process and interpret complex data obtained within metabolomic-based studies, advanced data mining/data processing software algorithms and multivariate chemometric tools are needed. Fortunately, mass-spectrometer manufacturers, but also research teams, have developed a number of software tools to treat metabolomic and proteomic research MS data. Instrument vendors provide software packages that are linked to the corresponding analytical platform of the instrument vendor (e.g., AB SCIEX provided MarkerView, PeakView, ProteinPilot and LipidView *etc*) (Theodoridis *et al.* 2008). One of the most popular data processing techniques in LC-MS based metabolomic study is principal component analysis (PCA) (Zhou *et al.* 2012). It is most frequently employed method for initial exploration of data internal structure and sample clustering (Vaclavik *et al.* 2012).

In this study, high performance liquid chromatography-mass spectrometry (HPLC-MS) technique, employing a hybrid triple quadrupole/time of flight (QTOF) mass analyzer, was used for comparative metabolomic fingerprinting of seven edible mushroom varieties, with the aim of identifying unique biomarker(s) from *Laetiporus sulphureus* which might be responsible for the pharmacological claim of the mushroom by the Kaffa people in Ethiopia.

2. Materials and Methods

2.1. Chemical and Reagents

HPLC grade methanol, acetonitrile, formic acid were used for sample extraction and mobile phase. Ultra-pure water (18.2 M Ω .cm) was used. Authentic standard of 18 α -Glycyrrhetic acid (Enoxolone) was purchased from Sigma Aldrich (Bellefonte, Pennsylvania).

2.2. Experimental Design

A comparative experimental design for seven mushroom samples each run in three replicates was used. *Laetiporus sulphureus* was compared against the other six edible mushrooms samples of Ethiopia collected from different part of country. A pilot test was conducted to evaluate LC-MS data variations under specific experimental conditions. The result from the pilot study was used to guide the subsequent experiments. To control the data quality, a blank was prepared and run in randomized order with the samples.

2.3. Samples Analyzed

The samples consist of *Pleurotus ostreatus*, *Lentinus edodes*, *Agaricus campestris*, *Laetiporus sulphureus*, *Termitomyces clypeatus*, *Termitomyces microcarpus* and *Termitomyces letestui* were selected on purpose for this comparative metabolomics. The samples were coded based on their common name as Oyster, Shiitake, *Agaricus*, *Laetiporus*, *Clypeatus*, *Microcarpus* and *Letestui*, respectively.

2.4. Metabolite Extraction

For the pilot test, only 20 mg of samples were extracted with 100 μ l of cool methanol and the extract was directly injected in to the LC-QTOF-MS. But for the actual test, samples were extracted based on the procedures previously outlined (Barros *et al.* 2007). Briefly, ten gram of dried mushroom powder was extracted by stirring with 100 ml of methanol at 25 $^{\circ}$ C at 150 rpm for 24 h using temperature shaker incubator and then filtered through Whatman No. 4 paper. The residue was then extracted with two additional 100 ml portions of methanol as described above. The combined methanolic extracts were evaporated at 40 $^{\circ}$ C to dryness using rota-evaporator and re-dissolved in methanol at the concentration of 50 mg/ml and stored at 4 $^{\circ}$ C for further use.

2.5. LC-QTOF-MS Platform and Data Acquisition

Chromatographic separation was performed using Shimadzu HPLC system (Shimadzu, Kyoto, Japan). The HPLC system consisted of a binary pumping system: pump A (LC-20AD XR) and pump B (LC-20AT XR), a degasser (DGU-14A), an autosampler (SIL-20AC XR), and column oven (CTO 20A) and system controller (CBM-20A). A solvent system consisting of 0.1% formic acid in water (A) and acetonitrile containing 0.1% formic acid (solvent B) was used with the following gradient: starting with 3% B and installing a gradient to obtain 45% B at 10 min, 75% B from 12-17.5 min and finally 3% B at 18 min. The system controller was stopped at the end of 20 min. The solvent flow rate was 0.25 ml/min.

Samples (5 μ L) were injected on to a C18 reversed-phase column set at 55 $^{\circ}$ C. Mass spectrometric detection was performed with a quadrupole-TOF-MS (Triple TOF 5600, AB SCIEX, Concord, Canada) operated in the positive and negative mode with a Duo Spray ion source. Information dependent acquisition using a TOF-MS survey scan 100-1000 Da (100 ms) and up to 10 dependent TOF MS/ MS scans 50-1000 Da (100 ms) using Collision Energy (CE) of 45 V with Collision Energy Spread (CES) of \pm 30 V. Each sample was injected three times in positive and negative polarity in randomized order to avoid any possible effect of time-dependent changes in chemical fingerprints.

2.6. Data Pre-Processing and Statistical Analysis

MarkerView software (version 1.2.1, AB SCIEX, Concord, ON, Canada) was used for processing (data mining, alignment, normalization, and PCA) of the HPLC-QTOF full MS records. Moreover, to evaluate the high resolution (HR) MS and MS/MS mass spectra obtained within HPLC-QTOF-MS analyses and to estimate elemental formulae of pre-selected marker compounds, PeakView software (version 1.0 with Formula Finder plugin version 1.0, AB SCIEX, Concord, ON, Canada) was used.

2.7. Tentative Identification of Biomarker (s)

Tentative identification of the m/z value for a pre selected biomarkers were searched against the metabolite database, Metlin (<http://metlin.scripps.edu/>). The molecular ion having molecular weights within a 5 ppm tolerance range of the query molecular weights are retrieved from the data bases as putative identifications. As necessary, the comparison was extended to MS² to MS³ or MS⁴ level for more confident identifications of metabolites. The search was done only with protonated [M + H]⁺ and deprotonated [M - H]⁻ in the positive and negative ionization, respectively.

2.8. Biomarker Verification

To verify the mass-based search results, authentic standards of those putative identifications were subjected to MS and tandem MS experiments together with the sample of interest. By comparing the retention times or tandem MS spectra of the authentic compounds with the ions of interest in the sample, the identities of the metabolites were confirmed. At least two independent and orthogonal data (retention time and mass spectrum, accurate mass and tandem mass spectrum etc) relative to an authentic compound analyzed under identical experimental conditions are used to verify putative identifications. However, due to time, cost and incompleteness of the online data base not all pre-selected biomarkers were identified.

2.9. Possible Mechanism of Action and Fragments

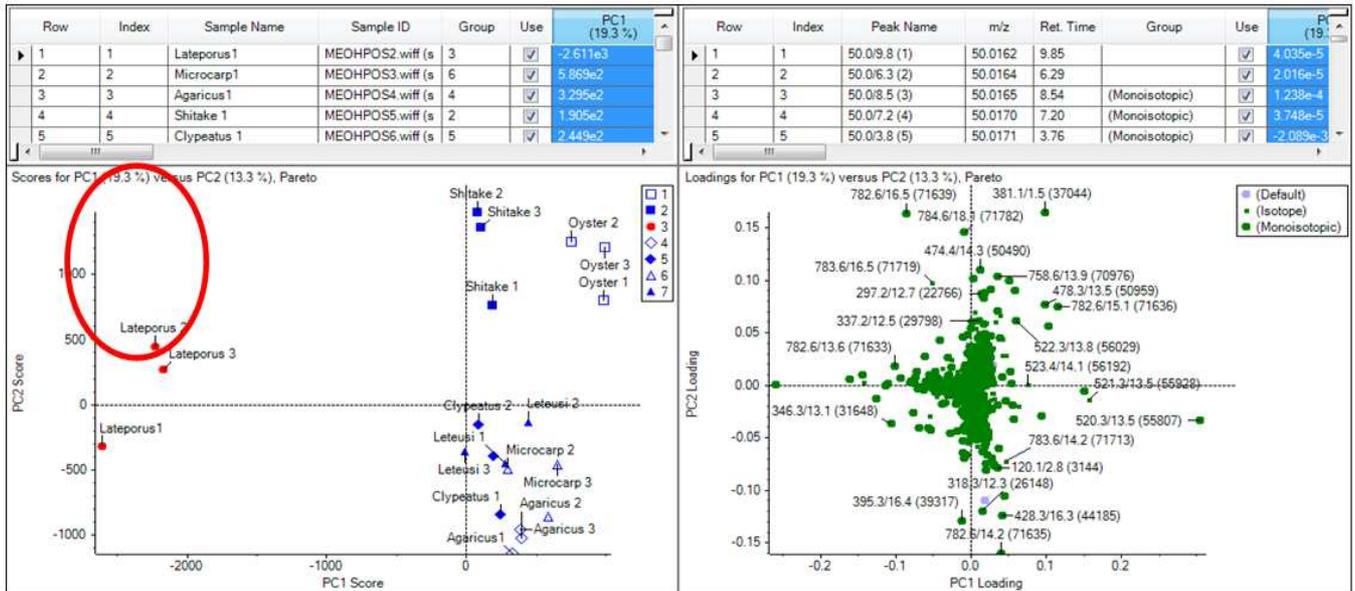
Mass Frontier (Version 7.0, HighChem, Ltd. Slovak Republic) was used for the management, evaluation and interpretation of the mass spectra of the verified molecular ion. The structure editor module of the software was first used for drawing the structure of the verified biomarker. Based on the chemical structure that was created, the fragments and mechanisms module of the software was used to provide information about the basic fragmentation and rearrangement mechanisms for the chemical structure. This module generates the possible fragments based on a set of known reaction mechanisms and library mechanism that support automated prediction.

3. Results and Discussion

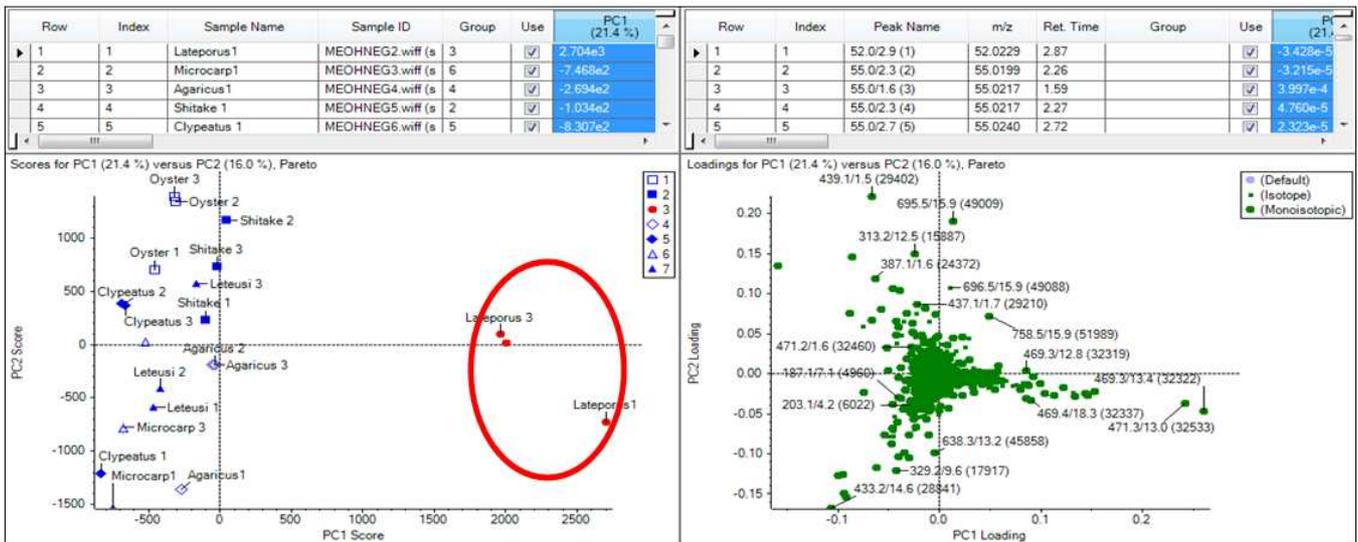
3.1. Alignment, Filtering and Normalization

First data were transferred from the instrumental computer to the processing computer at the Metabolomics Center of the Huck Institute of Life Science of Penn state University. The LC-MS data (as wiff file) were loaded into the MarkerviewTM software and adjusted for alignment and filtering using the peak finding options of the software. Two separate positive and negative ionization data matrices, comprising lists of peaks characterized for each sample by RT, m/z, intensity and charge state, were obtained. As an outcome of the data mining and aligning procedures, positive and negative ionization data matrices of 71083 and 54856 peaks, respectively, were obtained for the seven mushrooms each in triplicate analyzed with LC-QTOF. These data were normalized using the normalization function of the software based on total area sums before performing principal component analysis (PCA).

3.2. Principal Component Analysis (PCA)



(a)



(b)

Fig. 1. PCA consisted of the scores table, scores plot, loading table and loading plot of the seven edible mushrooms in the positive ionization (a) in the negative ionization (b).

Principal component analysis represents a highly useful tool when interpreting complex multivariate data sets, as it allows dimensionality reduction and visualization of the information present in the original data in the form of a few principal components (PCs) while retaining the maximum possible variability (Berrueta *et al.* 2007). Large differences in abundance of input variables (peaks) can significantly affect the data variance determined within PCA. To modify the weights of respective variables, Pareto scaling (the square root of the standard deviation is used as the scaling factor) of the data was performed. This type of scaling, which enables reduction, but not complete elimination of input variables abundance differences, provides good results when applied to LC-MS data, as it reflects the fact that larger peaks can be generally considered more reliable, but all variables are

equivalent (Vaclavik *et al.* 2012).

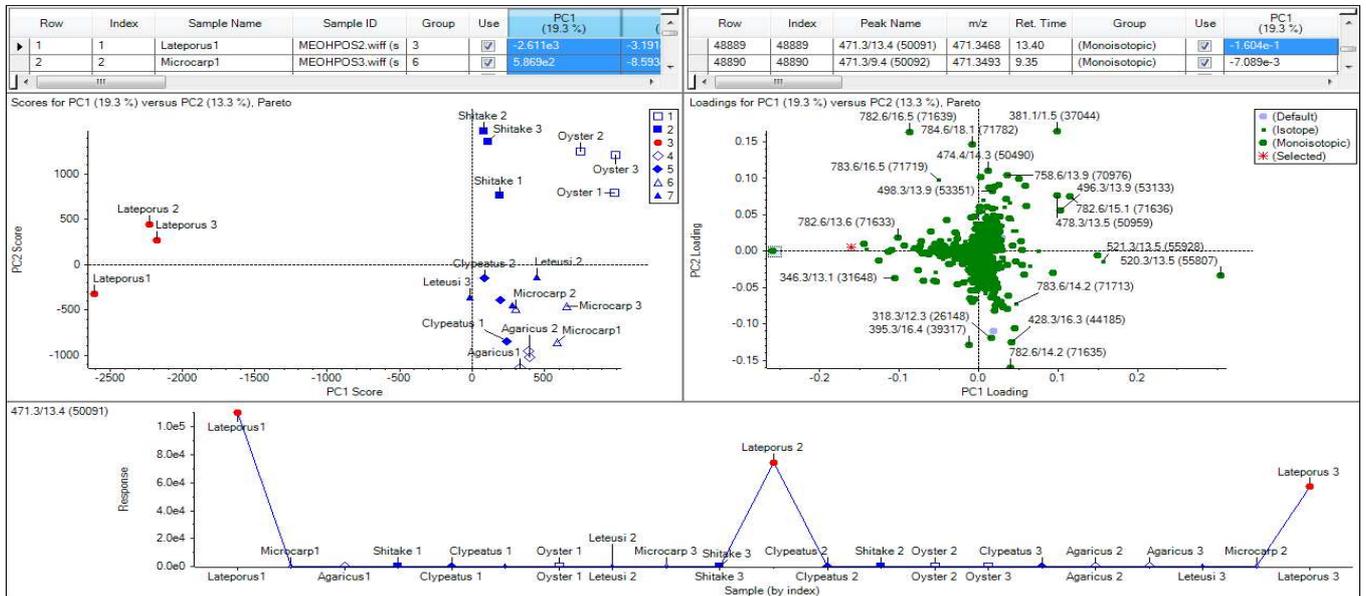
In this study also, PCA function of MarkerView™ software was used to get information on the mushroom samples clustering, as well as for selection of characteristic marker peaks of *Laetiporus sulphureus*. As show in Fig. 1a & b, regardless of the ionization mode, PCA of the data set representing the seven wild and cultivated mushrooms in each triplicate (n=3) revealed a unique separate clusters for *Laetiporus sulphureus*, documenting differences in LC-MS profiles associated with the sample.

3.3. Profile Plots

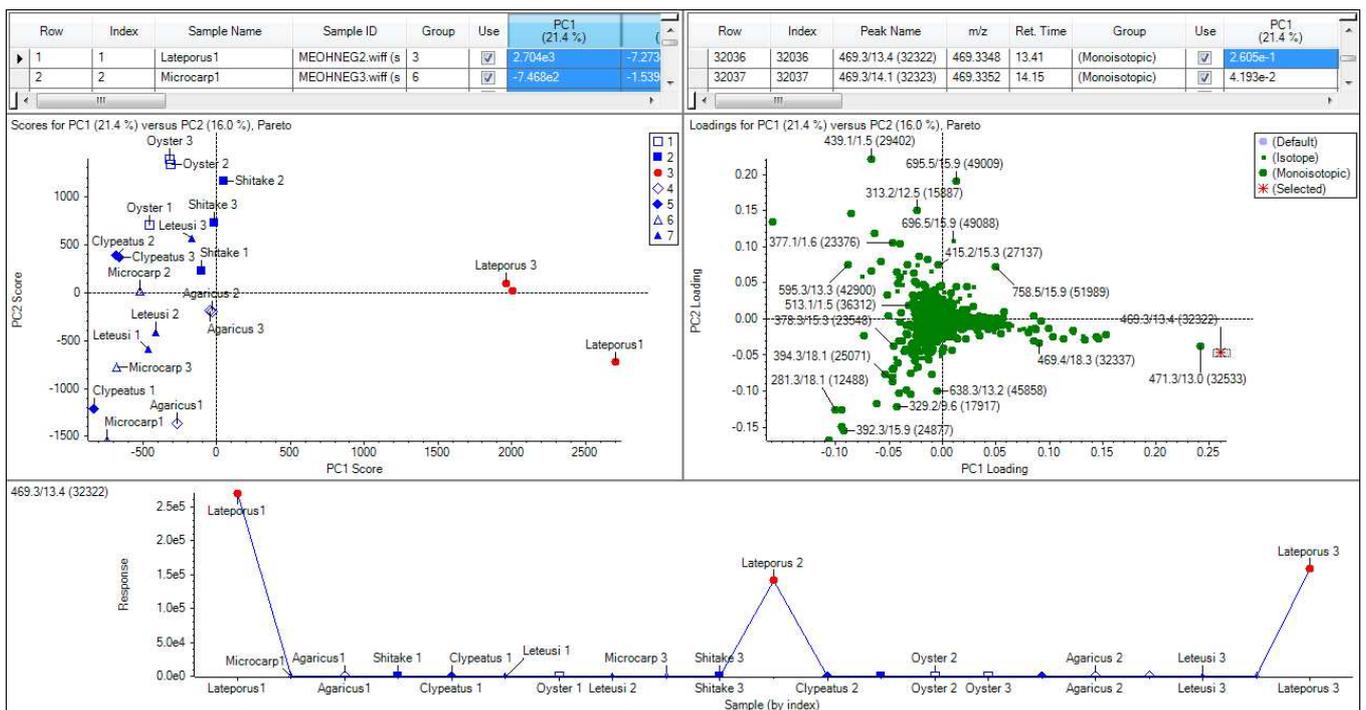
The profile plot in MarkerView™ software was used to verify that all variables of the selected group from the PCA

were characteristic for the *L.sulphureus*. Profile plots display peaks response as a function of sample for a given peak. These plots are more often used to focus on peaks likely to be of particular interest, such as the t-test table or PCA loading

plot (AB SCIEX 1010). Figures 2a &b elaborates procedures followed to plot profile of peaks of interest from the PCA loading plot of both negative and positive ionization mode, respectively.



(a)



(b)

Fig. 2. Plot profiling of one of the biomarkers unique to *Laetiporus sulphureus* at 13.4 min in the positive ionization (a) in the negative ionization (b).

3.4. Biomarkers (Peaks of Interest)

The combination of PCA and profile plots was able to reduce the list of peaks of interest (biomarkers) unique to *L.sulphureus* found by the non-target peak finding algorithm

from thousands to only a few dozen signals. Initially there were 16 pre-selected biomarkers in the positive ionization (Table 1), however only 14 peaks with monoisotopic mass were considered for further identification.

Table 1. Peaks of interest (Biomarkers) unique to *Laetiporus* in the positive ionization mode.

No.	Row	Index	Peak Name	m/z	Ret. Time	Group
1	19979	19979	284.1/3.7 (20696)	284.149	3.72	Monoisotopic
2	36978	36978	386.3/18.5 (37949)	386.3319	18.51	Monoisotopic
3	44377	44377	437.4/16.7 (45508)	437.341	16.7	Monoisotopic
4	44640	44640	439.4/17.2 (45777)	439.3562	17.2	Monoisotopic
5	44780	44780	440.4/17.2 (45919)	440.3606	17.2	Isotope
6	46260	46260	451.3/11.3 (46260)	451.3205	11.33	Monoisotopic
7	46264	46264	451.3/12.5 (47425)	451.3233	12.53	Monoisotopic
8	46515	46515	453.3/17.7 (47683)	453.3359	17.71	Monoisotopic
9	46519	46519	453.3/13.4 (47687)	453.3368	13.4	Monoisotopic
10	46795	46795	455.4/13.1 (47969)	455.3519	13.07	Monoisotopic
11	46927	46927	456.4/13.1 (48103)	456.3561	13.09	Isotope
12	48621	48621	469.3/11.3 (49815)	469.3312	11.33	Monoisotopic
13	48622	48622	469.3/12.4 (49816)	469.3318	12.4	Monoisotopic
14	48636	48636	469.4/13.4 (49830)	469.3659	13.4	Monoisotopic
15	48889	48889	471.3/13.4 (50091)	471.3468	13.4	Monoisotopic
16	48902	48902	471.4/18.7 (50104)	471.3822	18.67	Monoisotopic

Similarly, there were 35 pre-selected biomarkers in the negative ionization (Table 2), although only the 27 peaks had the monoisotopic mass. Each biomarker had ID based on retention time, m/z and their row number in the original data.

Table 2. Peaks of interest (Biomarkers) unique to *Laetiporus* in the negative ionization mode.

No.	Row	Index	Peak Name	m/z	Ret. Time	Group
1	26693	26693	413.3/14.2 (26936)	413.2546	14.17	Monoisotopic
2	29774	29774	445.3/10.1 (30042)	445.2983	10.1	Monoisotopic
3	30546	30546	453.3/16.6 (30822)	453.3411	16.59	Monoisotopic
4	30547	30547	453.3/18.2 (30823)	453.3417	18.19	Monoisotopic
5	30639	30639	454.3/16.6 (30916)	454.3448	16.59	Isotope
6	30641	30641	454.3/18.2 (30918)	454.3457	18.19	Isotope
7	30751	30751	455.4/17.1 (31029)	455.3572	17.07	Monoisotopic
8	30848	30848	456.4/17.1 (31126)	456.3613	17.07	Isotope
9	31080	31080	459.3/9.9 (31359)	459.2751	9.94	Monoisotopic
10	31838	31838	467.3/13.3 (32122)	467.3194	13.29	Monoisotopic
11	32033	32033	469.3/12.8 (32319)	469.3323	12.83	Monoisotopic
12	32036	32036	469.3/13.4 (32322)	469.3348	13.41	Monoisotopic
13	32037	32037	469.3/14.2 (32323)	469.3352	14.15	Monoisotopic
14	32428	32428	470.3/13.4 (32428)	470.3396	13.41	Isotope
15	32244	32244	471.3/13.9 (32532)	471.3494	13.87	Monoisotopic
16	32245	32245	471.3/13.0 (32533)	471.3498	13.03	Monoisotopic
17	32340	32340	472.4/13.0 (32629)	472.3543	13.03	Isotope
18	33177	33177	481.3/13.6 (33470)	481.3359	13.61	Monoisotopic
19	33363	33363	483.3/12.2 (33363)	483.3142	12.25	Monoisotopic
20	33377	33377	483.4/13.7 (33671)	483.3513	13.66	Monoisotopic
21	33475	33475	484.4/13.7 (33769)	484.3556	13.66	Isotope
22	33562	33562	485.3/11.3 (33857)	485.3299	11.34	Monoisotopic
23	33564	33564	485.3/12.5 (33859)	485.3306	12.49	Monoisotopic
24	33578	33578	485.4/13.4 (33873)	485.3642	13.41	Isotope
25	33660	33660	486.3/11.3 (33955)	486.3339	11.34	Isotope
26	33663	33663	486.3/12.4 (33958)	486.3347	12.43	Isotope
27	33673	33673	486.4/13.4 (33968)	486.37	13.41	Isotope
28	33760	33760	487.3/10.3 (34057)	487.343	10.27	Monoisotopic
29	33764	33764	487.3/13.1 (34061)	487.3453	13.06	Monoisotopic
30	33772	33772	487.4/13.3 (34069)	487.3636	13.28	Monoisotopic
31	33851	33851	488.3/10.4 (34148)	488.3496	10.44	Isotope
32	34652	34652	497.3/12.3 (34956)	497.3302	12.29	Monoisotopic
33	34930	34930	500.4/12.3 (35235)	500.3501	12.3	Isotope
34	35026	35026	501.4/11.5 (35331)	501.3607	11.55	Monoisotopic
35	35179	35179	503.3/10.0 (35484)	503.3372	9.99	Monoisotopic

3.5. Tentative Identification of Marker Compounds

The identification of discriminating marker compounds represents probably the most laborious and time-consuming step of the metabolomic workflow. The use of a high-resolution instrument enabling to obtain both single MS and

MS/MS accurate mass spectra is needed for reliable elemental formula estimation, which is typically followed by a database search (Vaclavik *et al.* 2012). First, the m/z value of a molecular ion of interest is searched against database(s) (Wishart *et al.* 2007; Cui *et al.* 2008; Smith *et al.* 2005; Go

2010). The molecules having molecular weights within a specified tolerance range to the query molecular weight are retrieved from databases as putative identifications. In this study, search for the pre-selected biomarkers of *Laetiporus sulphureus* was performed from the METLIN online data base (<http://metlin.scripps.edu/>) in the positive and negative mode by protonation $[M+H]^+$ and deprotonation $[M-H]^-$, respectively.

Each 14 and 27 pre-selected peaks were searched from Metlin based on their m/z, followed by MS and MS/MS comparison. However, only one biomarker with m/z of 471.3468 and 469.3348 at the retention time of 13.41 in the positive and negative mode was tentatively identified as glycyrrhetic acid (Enoxolone), respectively. Generally less than 30% of the detected ions in a typical LC-MS-based metabolomic experiment can be uniquely identified through mass-based search, leaving most of the ions either unidentified or with multiple putative identifications. Improved approaches, such as those involving isotope labeling, can be used to reduce the ambiguities from the mass-based search. But they cannot guarantee unique identification either (Giavalisco et al. 2009).

3.6. Tandem MS Comparison between Sample and Database

The information dependent acquisition (IDA) display allows MS/MS spectra (and possibly MS³) which are collected automatically when data in one or more survey spectra meet certain criteria. The PeakView™ Software contains numerous tools to display, filter, and process IDA data. When figure indicates that mass/retention time is unique to group, the peak was opened in peak view for further analysis (AB SCIEX, 2012). Hence, from IDA explorer of the software, the data point on spectrum that is closest to the mass/retention values from the loading plot was selected.

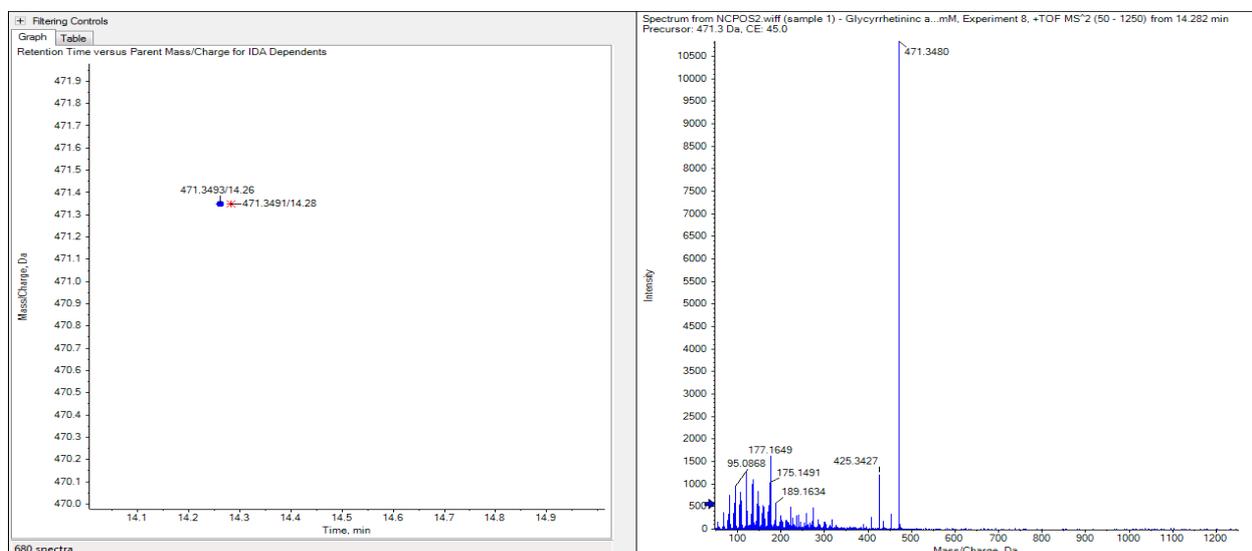
PeakView software also allows comparing fragments from online database search with accurate mass MS/MS information to further increase confidence in identification (Vaclavik et al. 2012) Hence; PeakView provided the tandem

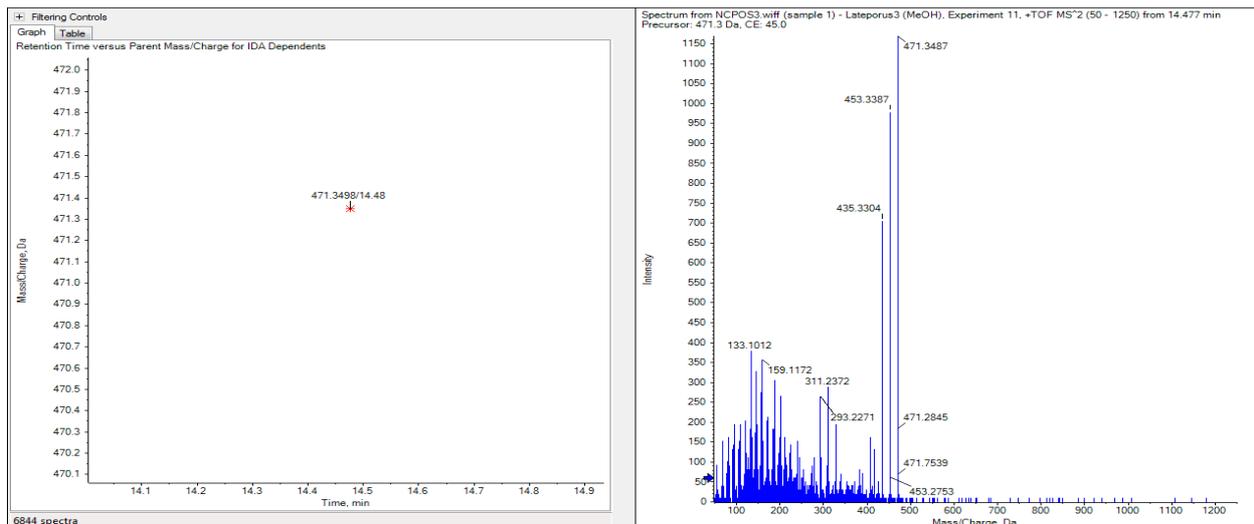
MS spectra for m/z of 471.3460 in the positive mode. After logging onto <http://metlin.scripps.edu/> to further analyze the data and attempt to identify the compound as a potential biomarker. The tandem MS spectra provided by the data base for glycyrrhetic acid at different energy of ionization were also evaluated.

3.7. Verification of Metabolite (Marker)

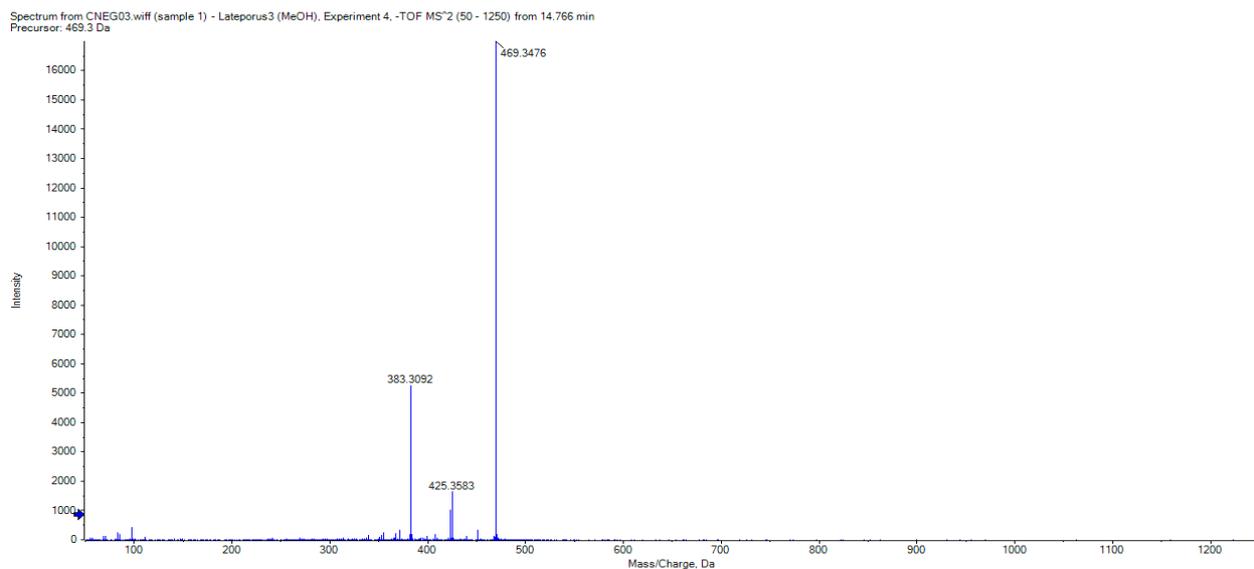
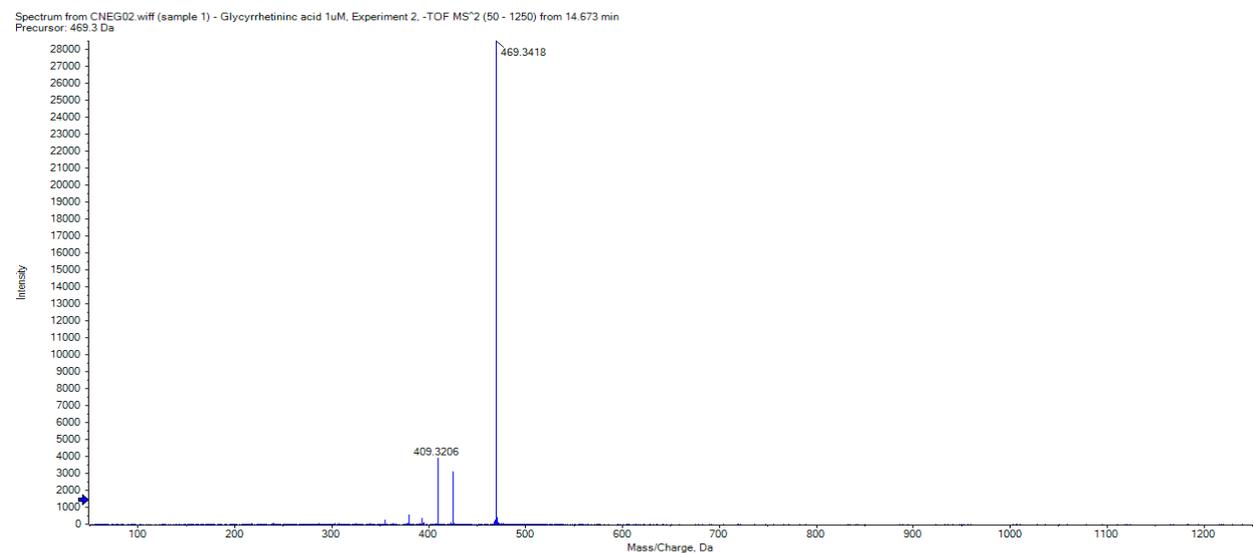
To verify the mass-based search results, authentic compounds of those putative identifications must be subjected to MS or tandem MS experiments together with the sample. By comparing the retention times or tandem MS spectra of the authentic compounds with the ions of interest in the sample, the identities of the metabolites can be confirmed. It may be necessary to extend the MS² to MS³ or MS⁴ level for more confident identifications of some metabolites. It was suggested that at least two independent and orthogonal data (retention time and mass spectrum, accurate mass and tandem mass spectrum, etc.) relative to an authentic compound analyzed under identical experimental conditions are necessary to verify a putative metabolite identification (Sumner et al. 2007).

In this study, to verify the mass-based search result of the putative identification, authentic compound of 18 α -glycyrrhetic acid (enoxolone) was purchased from Sigma-Aldrich and subjected to MS and tandem MS experiments together with the sample. As shown in Fig. 3a & b, by comparing the retention time, MS and MS/MS spectra of the authentic standard of enoxolone with the *Laetiporus* sample, the identity of the putative was somewhat confirmed. The only change in retention time from the original data was due to the application of a different column. According to Zhou et al. (2012) the limiting factor of verification is that it is often costly and time consuming. The authentic compounds of putative identifications need to be acquired. More experiments need to be performed. Sometimes, a molecular ion can have more than 100 putative identifications which make manual verifications extremely laborious.





(a)



(b)

Fig. 3. Metabolite ID verification by comparison of the retention times, MS and MS/MS spectra of the authentic standard of 18 α -glycyrrhetic acid (TOP) and experimental sample *Laetiporus sulphureus* (Bottom) in positive ionization (a) and in negative ionization (b).

3.8. Glycyrrhetic Acid (Enoxolone)

According to literatures glycyrrhetic acid or commonly called enoxolone is the active form of glycyrrhizin which is the major pentacyclic triterpene in licorice (*Glycyrrhiza glabra L.*). It has been shown to possess several pharmacological activities, such as anti-ulcerative, anti-inflammatory, immunomodulating (Chung *et al.*, 2001) and anti-tumour activities (Ryu *et al.*, 1994; Luo *et al.*, 2004). In addition, direct and indirect antiviral activity, interferon-inducibility and anti-hepatitis effects have been attributed to glycyrrhetic acid (Barran *et al.*, 1974; Mahato *et al.*, 1992). It is extensively used in Japan and has been examined in Europe in patients with acute and chronic hepatitis to reduce the progression of liver disease to hepatocellular carcinoma (Arase *et al.*, 1997; Van Rossum *et al.*, 1998). A recent report showed that glycyrrhetic acid also reverses the multidrug resistance to cancer chemotherapeutic agents and can be considered as a promising lead compound for the design of more efficacious and less toxic chemosensitizing agents to enhance the efficacy of cancer chemotherapy (Nabekura *et al.*, 2008).

According to Chandler (1985) Glycyrrhetic acid also has expectorant and antitussive properties. Expectorants are used to decrease the viscosity of tenacious mucus, or to increase the secretion of mucus in dry irritant unproductive cough, thereby, lubricating the air passages and making coughing more productive. It is used considerably as a flavoring agent and is frequently employed to mask the taste of bitter drugs such as aloe, quinine etc.

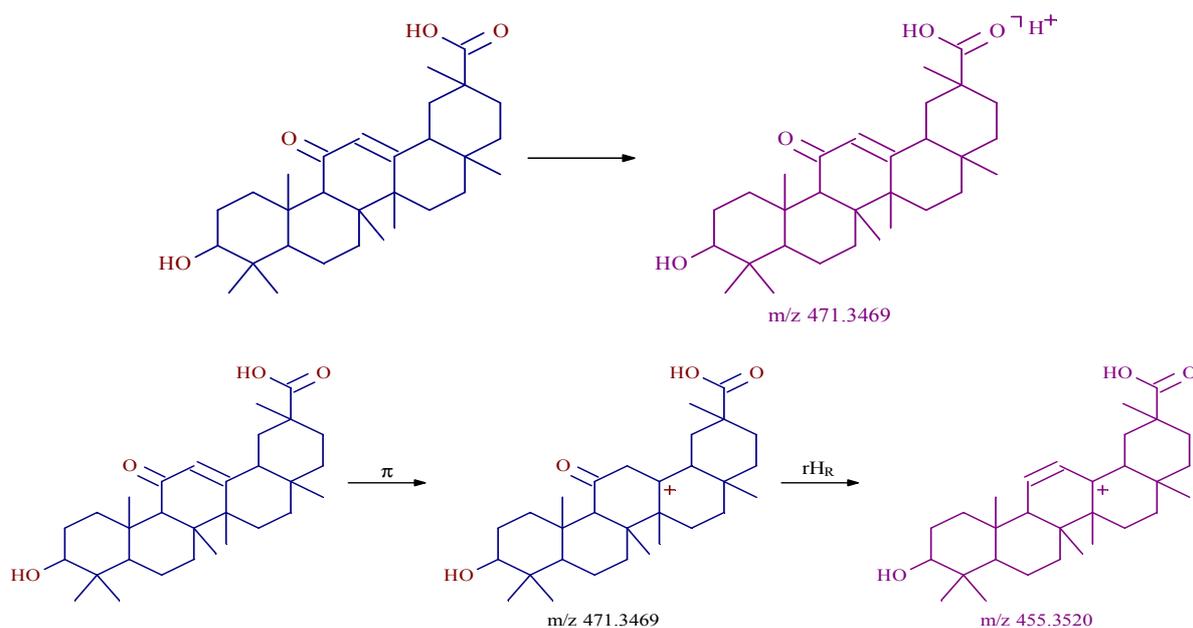
Glycyrrhetic acid inhibits the enzymes (15-hydroxyprostaglandin dehydrogenase & delta 13-prostaglandin) that metabolise the prostaglandins, PGE₂ and PGF_{2alpha} to their respective 15 keto-13, 14-dihydro metabolites which are inactive. This causes an increased level of prostaglandins in the digestive system. Prostaglandins

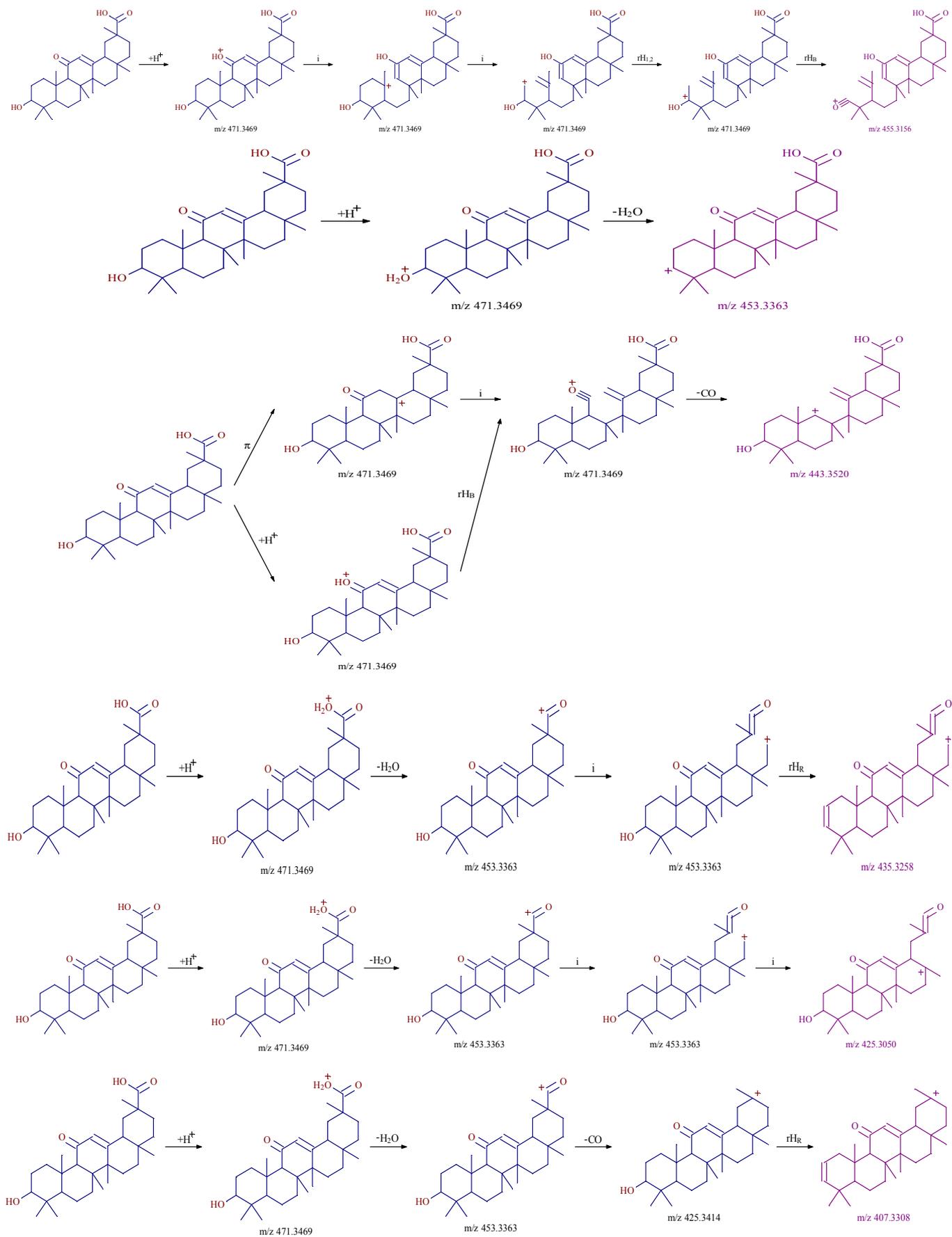
inhibit gastric secretion but stimulate pancreatic secretion and mucous secretion in the intestines and markedly increase intestinal motility. They also cause cell proliferation in the stomach. The effect on gastric acid secretion, promotion of mucous secretion and cell proliferation shows why licorice has potential in treating peptic ulcer (Baker, 1994). PGF_{2alpha} stimulates activity of the uterus during pregnancy and can cause abortion; therefore, licorice should not be taken during pregnancy (Strandberg *et al.*, 2002).

Thus, the ethnopharmacological information obtained from the kaffa people with regards to *L.sulphureus*' health benefits such as muscle relaxation and relief for stomach pain might be true. Although further, in vivo or animal studies should be carried to close the loop. Moreover supportive informations from other metabolomics platforms such as NMR, GC-MS and other should be evaluated to identify the unidentified biomarkers in *L.sulphureus* or the other six mushrooms evaluated in this study. To our knowledge, no study has isolated glycyrrhetic acid from other plant source or fungi except licorice roots till now.

3.9. Possible Metabolic Pathways for Major Fragments of Glycyrrhetic Acid

Mass Frontier (Version 7.0, HighChem, Ltd. Slovak Republic) was used for the management, evaluation and interpretation of the mass spectra of the verified molecular ion. The structure editor module of the software was first used for drawing the structure of the verified biomarker. In this study, the fragments and mechanisms module of the software provided 89 (positive mode) and 3 (negative mode) unique theoretical fragmentation from 18 α - glycyrrhetic acid after different rearrangement mechanisms for the chemical structure. Fig. 4a & b depicts some of the theoretical fragments generated by the software from the positive and negative m/z spectra of 18 α - glycyrrhetic acid.





(a)

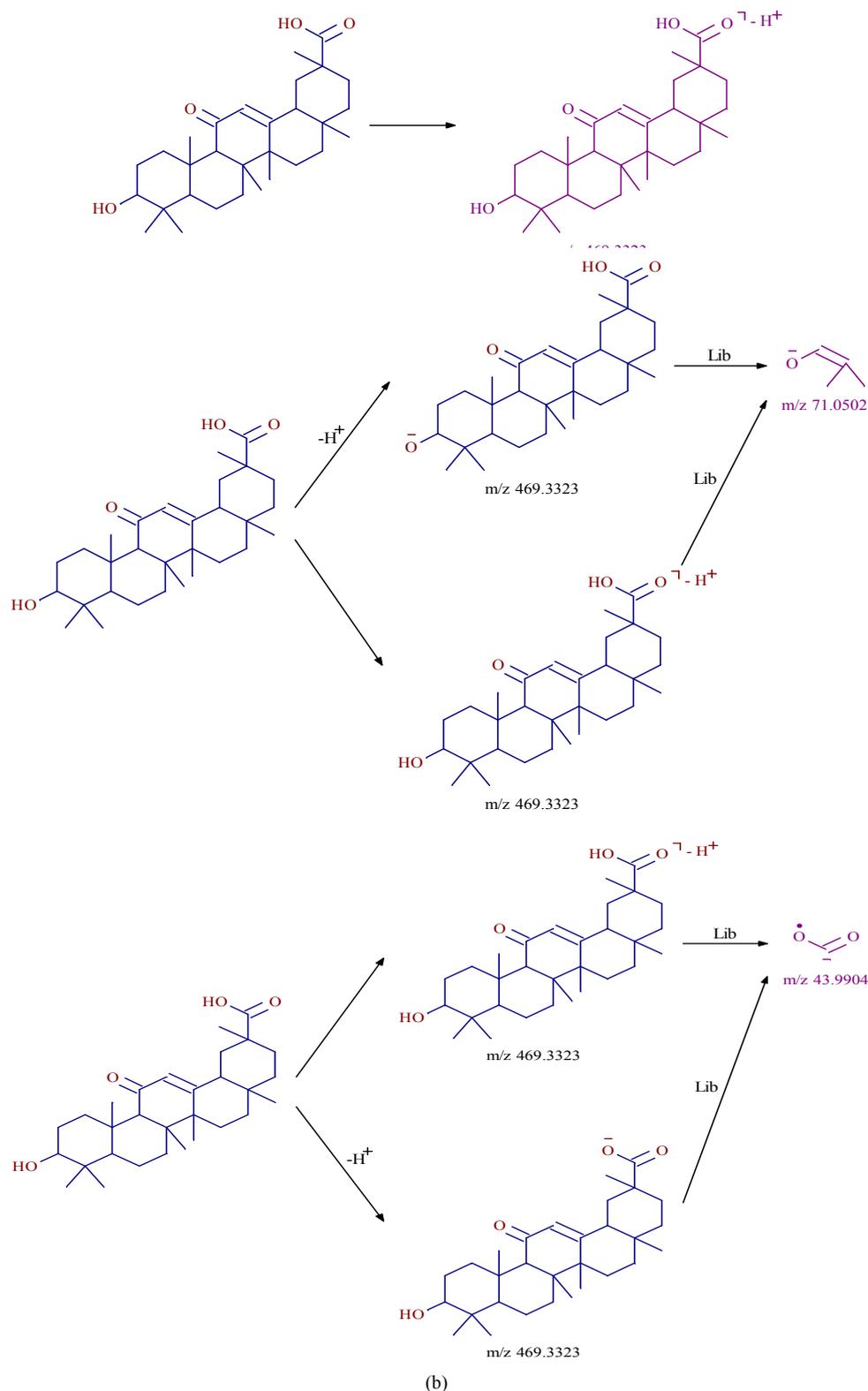


Fig. 4. Possible mechanisms for some fragments provided by mass frontier 7.0 software in the positive ionization (a) and the negative ionization (b).

4. Conclusions

Glycyrrhetic acid (Enoxolone) might be one of the biomarker responsible for ethnopharmacological health benefits suggested by the kaffa people with regards to

L.sulphureus mushroom. However, further in vivo or animal studies and identification of the unidentified pre-selected biomarkes in both the positive and negative ionization mode should be carried to close the loop. Moreover supportive informations from other metabolomics platforms such as

NMR, GC-MS and other should undertaken. It is also possible to re-interrogate the huge metabolomic data as new hypotheses posed about the seven edible mushrooms of Ethiopia with no further sample analysis.

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