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NMR spectroscopy and mass spectrometry in metabolomics analysis of *Salvia*

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Abstract The interest in using the '-omics' approach for nutrition, agriculture, food science and human health have seen an explosive growth in the last years. Particularly, metabolomics analysis is becoming an integral part of a system biological approach for investigating organisms. In this review, the limitations and advantages of NMR spectroscopy and mass spectrometry were discussed in details using the study reported in the literature on different *Salvia* species (*S. hispanica, S. miltiorrhiza, S. officinalis, S. runcinata* and *S. stenophylla)*. Both approaches identify and quantify several classes of compounds but not the complete metabolite profile of the plant. A combined approach of these two powerful techniques provides better results allowing to determine both primary and secondary metabolites.

Keywords Metabolomics · *Salvia* · Sage · NMR · MS · Multivariate data analyses

Introduction

Over the past few years, the '-omics' fields have seen an explosive growth opening new perspectives for biological research purpose. The development of analytical instrumentations, data processing and chemometric tools simplify the study of complex biological systems on a large-scale. Metabolomics, together with other '-omics' disciplines such as genomics, transcriptomics, and proteomics, is becoming an integral part of a system biological approach for investigating organisms. Figure 1 reports the classification of the '-omics' technologies and the correlation among them. Although transcriptome represents the process for protein synthesis, an increase in mRNA levels does not always correspond to an increase in proteins due to a number of post-transcriptional regulation mechanisms (Kendrick 2014; Vogel and Marcotte 2012). Therefore, changes in transcriptome or proteome do not always reflect alterations in biochemical phenotypes. For this reason, the association of metabolomics to the other analytical areas of genomics, transcriptomics and proteomics constitute a very powerful tool to study biological systems.

Metabolomics is the '-omic' studying the whole metabolome in a cell, tissue or organism from both qualitative and quantitative point of view. The interest in using metabolomics for nutrition, agriculture, food science, human health and drug discovery has seen an exponential increase in the last years. In fact, the number of publications containing the term "metabolomics" is constantly growing.

In the whole metabolome, there are two groups of compounds, primary metabolites and secondary ones. Primary metabolites are ubiquitous compounds

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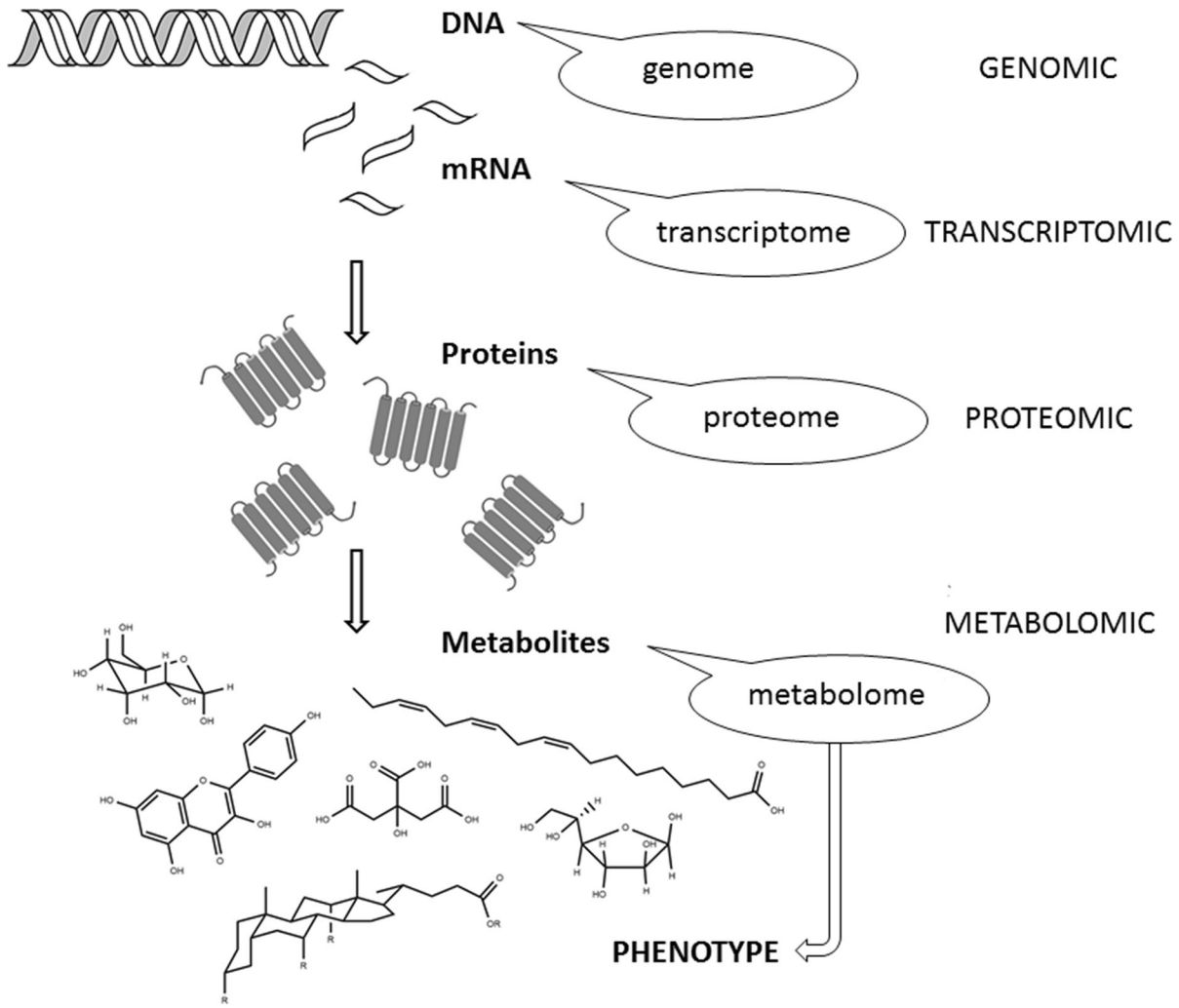

Fig. 1 General classification of '-omics' technologies

involved in the basic functions such as respiration, growth and maintenance of a cell. Basically, all organisms share the same type of primary metabolites. Secondary metabolites are non-ubiquitous and thus species specific compounds playing a role in the interaction of a cell with other cells or with the environment. Secondary metabolites are responsible of plant flavours and colours and are associated to plant resistance against pests and diseases.

The chemical complexity of metabolites ranges from ionic inorganic species to complex organic products, from hydrophilic to lipophilic compounds, from volatile to non-volatile molecules and from low to high molecular weight metabolites. Their production is not only regulated by gene expression but also by environmental conditions and it is for this reason that the metabolomics is able to define the biochemical phenotype of the studied subject.

During the metabolomics analysis, both primary and secondary metabolites will be detected after a snapshot of the entire metabolome and relative or absolute quantification can be done. This approach is applied on several topics which include:

- Studying fingerprints of different species, varieties, genotypes or ecotypes to obtain more information about taxonomy or biochemistry (Brahmi et al. 2015; de Falco et al. 2016).
- Comparing several classes of metabolites in response to external chemical or physical treatments (Catola et al. 2016; de Falco et al. 2017b).

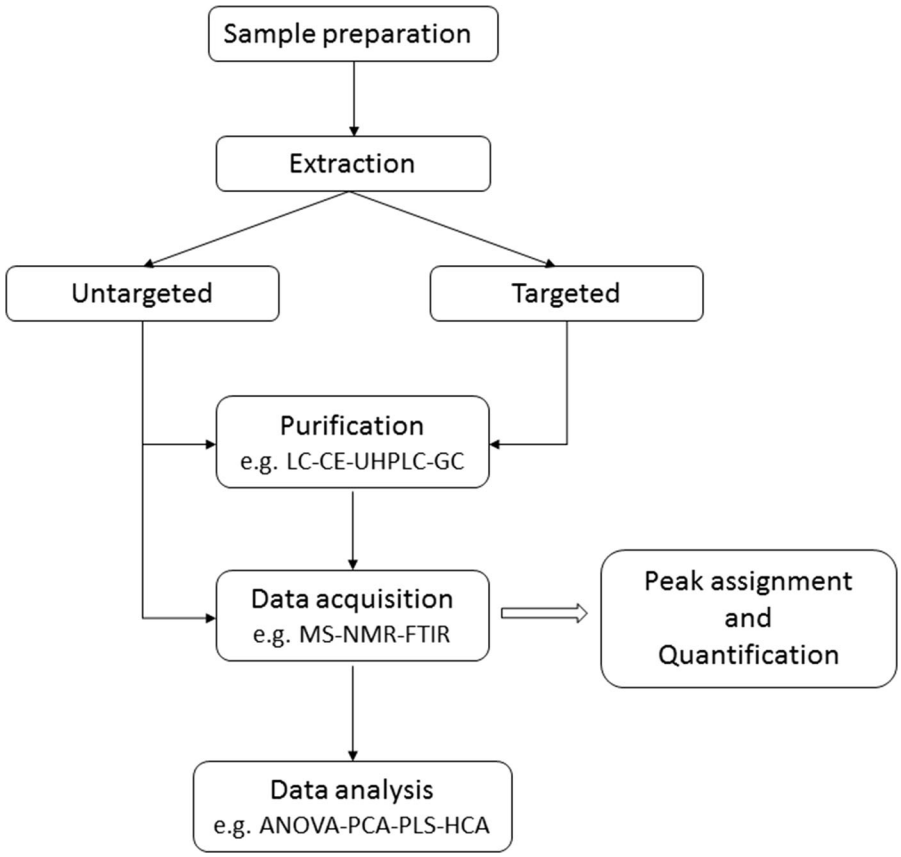

Fig. 2 Simplified workflow of the process for metabolomics analyses

- Highlighting differences and similarities between the metabolite content of mutants or transgenic plants and their wild-type counterparts (Kristensen et al. 2005).
- Monitoring developmental processes such as metabolic transition from immature to ripe fruit (Aharoni et al. 2002).

Generally, in the metabolomics studies there are four critical steps (Fig. 2). The first one is sample preparation. At this step, it is necessary to control time and temperature which influence the accuracy and the reproducibility of results. Metabolic reactions are extremely rapid, so the freezing and the storage of samples at − 80 °C are required.

The second step is the extraction procedure aiming to maximize the number and the content of metabolites of interest. A recent study on *Arabidopsis thaliana* showed that a particular combination of $MeOH/H_2O/CHCl_3$ is the best solvent mixture in comparison to others (Gullberg et al. 2004). Ultrasonic treatment is also used as a disruption method to reduce the time and to improve the efficiency of the extraction.

Following this, a third step is the use of several detection techniques to analyse the extracts. A high amount of information is generated by analytical data acquisition and compounds identification; therefore, data analysis is the fourth crucial step, briefly discuss in paragraph 1.3. A further separation or purification of metabolites after their extraction is also possible, but in many applications, samples are analysed directly. Whether this intermediate step is necessary, depends on the kind of research that is done. Nowadays, two main strategies are used for metabolomics investigations: untargeted (non-targeted) metabolomics and targeted metabolomics. The untargeted approach aims to compare the whole metabolite profiles among different sample groups, whereas targeted metabolomics focuses on a tiny fraction of

the metabolome analysing a specific group of compounds related to a specific metabolic pathway (e.g., fatty acids, amino acids or phytochemicals) (Son et al. 2008; Vrhovsek et al. 2012). For this purpose, one or few metabolites are selected after a high number of purifications and all other compounds are ignored. This approach is used to study know compounds and to get general information on the presence of unknown compounds in the analyzed material. However, to determine the chemical structure of compounds never reported in the literature it is necessary to obtain them as pure metabolites and to subject them to the classic spectroscopic and chemical analytical method.

Metabolomics aims to identify and quantify the overall metabolome. To achieve this objective, several analytical approaches can be used, each of them with advantages and disadvantages. They can be grouped as follows:

- chromatographic methods: liquid chromatography (LC), high-performance LC (HPLC); ultra-high performance LC (UHPLC), gas chromatography (GC), capillary electrophoresis (CE), thin layer chromatography (TLC);
- mass spectrometry (MS);
- spectroscopy: nuclear magnetic resonance spectroscopy (NMR); fourier transform infra-red (FT-IR); ultraviolet methods (UV).

Each technique, mentioned above, can be applied at two different levels. The first level is a qualitative study in which the signals observed are assigned to specific metabolites with the help of standard compounds, data literature and different libraries (Smith et al. 2005; http://www.hdscience.com). Consequently, the second level of analysis is the measurement of relative or absolute amounts of the detected components based respectively on spectra integration or calibration curves of internal standard. A compromise between speed, selectivity and sensitivity should be found between all these methods to select the most suitable approach, although a combination of two or more of them is also used to provide complementary information and to reduce sample complexity. For example, LC/MS with atmospheric pressure ionization (API) is also applied to metabolomics study, but only a relatively small number of analytes can be detected. Specifically, the production of pseudo-molecular ions ($[M + H]^+$ or $[M - H]^-$) depends on several factors and it is not always easy to predict which one will be produced. In addition, many compounds do not ionize optimally and for this reason LC/MS is more suited for metabolites which ionize similarly under the same condition. A rapid and non-destructive technique is the FT-IR, based on the stretching and bending vibration of chemical bonds irradiated by the light (usually 4000–400 cm^{-1}). Although it does not require a complex sample preparation, its sensitivity is not as high as that of the other techniques. However, in the last few years, several studies have been undertaken using FT-IR to diagnose disease or dysfunction and to acquire metabolic profiling of body fluids (Lemes et al. 2016; Isogawa et al. 2014). On the contrary, in other research fields, such as plant science and food chemistry, metabolomics studies by NMR and MS have been mostly applied. This review assesses the limitations and advantages of NMR spectroscopy and gas chromatography–mass spectrometry in metabolomics analyses, providing a description of these analytical techniques and their application to the metabolite profiling of *Salvia* species.

NMR based metabolomics

In detail, NMR spectroscopy is a physical measurement of the resonances of atoms with a non-zero magnetic moment, such as ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P in a strong magnetic field. The application of a magnetic field on the nuclei of these atoms causes the promotion of electrons from low-energy to high-energy spin states and the subsequent emission of radiation during the relaxation process. It is a non-destructive technique with high reproducibility in which compounds and extracts have a highly specific spectrum. Its sensitivity can be improved by the application of high and uniform magnetic fields (frequencies commonly used range between 300 and 700 MHz). The sensitivity of NMR also depends on the abundance of isotope studied and time of analysis. The most sensitive isotopes are ^{1}H, ^{19}F and ^{31}P, but the last two nuclei can only be found in a restricted number of compounds. For this reason, ^{1}H is the preferred nuclei for metabolites fingerprinting studies. The only variables are the solvents used which depend on the polarity of the extract analysed. In the ^{1}H NMR sample there is always much more solvent than substance, so deuterated solvents, such as D_2O, CD_3OD and $CDCl_3$, are

used to minimize the solvent signal in the spectra. During the spectrum acquisition, pH and temperature must be controlled to avoid shifts of the proton signals. Normally, the temperature is set on the spectrometer before the start of sample acquisition, while pH is kept constant by the use of a buffering agent, such as KH_2PO_4, and sodium deuteroxide. The chemical shifts in ^1H NMR spectra (0–10 ppm) are assigned to a specific metabolite. The major limitation of the NMR spectroscopy is the low sensitivity. Therefore, larger amounts of sample are required for NMR analyses than those required for other techniques. However, the introduction of the new analytical instrumentations, such as the use of microprobe and cryoprobe, improves the detection of signals that are not normally detected with a conventional probehead. In the first case, using a 1 mm microprobe it is possible to analyse very small quantities of a sample diluted in 10 µl rather than 500 µl (5 mm) to get a better quality of the spectra. Whereas, in the second case the probehead is cooled down to 20 K with a cryogen (liquid helium) to increase the signal to noise ratio.

The signals can be reported along two frequency axes to produce a two-dimensional spectrum (2D) which can be either homonuclear, if the correlation comes from the same nucleus (usually ^1H–^1H), or heteronuclear if on the frequency axes there are two different nuclei (usually ^1H–^{13}C). The 2D-NMR experiments are very useful in the case of an extensive signal overlap in some regions of the proton spectra, such as sugar region.

After spectra acquisition and compounds identification with pure standard and data from literature, the quantification is performed. Usually, DSS (dimethyl-4-silapentane sodium sulfonate) and TMS (tetramethylsilane) are used both as reference for the calibration of NMR shifts (resonating at 0.00 ppm) and as internal standard, for aqueous and organic fractions, respectively.

The integrated area under the peaks is calculated and compared to the peak intensity of the internal standard. Certain regions of the spectrum, such as those containing solvent signals, are typically excluded from the binning process.

Once completed the identification and quantification analyses, the statistical analyses can be applied to interpret the huge amount of data.

MS based metabolomics

A more sensitive tool is GC–MS, which is widely applied in metabolomics because of its high specificity and sensitivity for suitable analyte classes. This technique, similarly to LC/MS, is based on the separation and detection of ions, according to their mass–charge (*m*/*z*) ratio. A limitation of gas chromatography coupled with mass spectrometry is that not all injected components will pass through the column, because of different physico-chemical properties of the analytes (polarity, stability, molecular mass, volatility, etc.). Several components therefore remain in the injector or in the column, causing the whole system to respond differently after each injection. However, this can be avoided if all samples are derivatized at room or higher temperature before the analysis in order to reduce their polarity. After derivatization, the volatility and thermal stability of the compounds are thus provided, allowing their elution at high temperatures without decomposition.

In particular, compounds containing active hydrogens, such as –OH, –SH, –NH and –COOH should be derivatized prior to GC–MS analysis. Derivatization can be achieved by three general reactions, which are alkylation, acylation and silylation (Orata 2012). Usually, silylation is the most suitable reaction for non-volatile compounds for GC analysis. As shown in Fig. 3a, b, after silylation, an active hydrogen is substituted by a silyl group. It is important to underline that, despite derivatization, still numerous compounds will not pass through a GC-column due to their high molecular weight. Moreover, an oximation reaction is recommended, prior to silylation, in polar extracts containing sugars. It is well known that sugars like fructose and glucose have different chemical structures in water solution with the predominance of cyclic forms. To reduce the tautomerism, which can produce multiple peaks for the same compound on the chromatogram, the oximation is required (Shepherd et al. 2007) thus converting the carbonyl groups of aldehydes and ketones into oximes (see the reaction in Fig. 3c, d). There are several oximation and silylation reagents which can be used, but recent studies have shown that methoxyamine hydrochloride and *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide are the most appropriate for metabolomics studies (Dettmer et al. 2007; Ruiz-Matute et al. 2011).

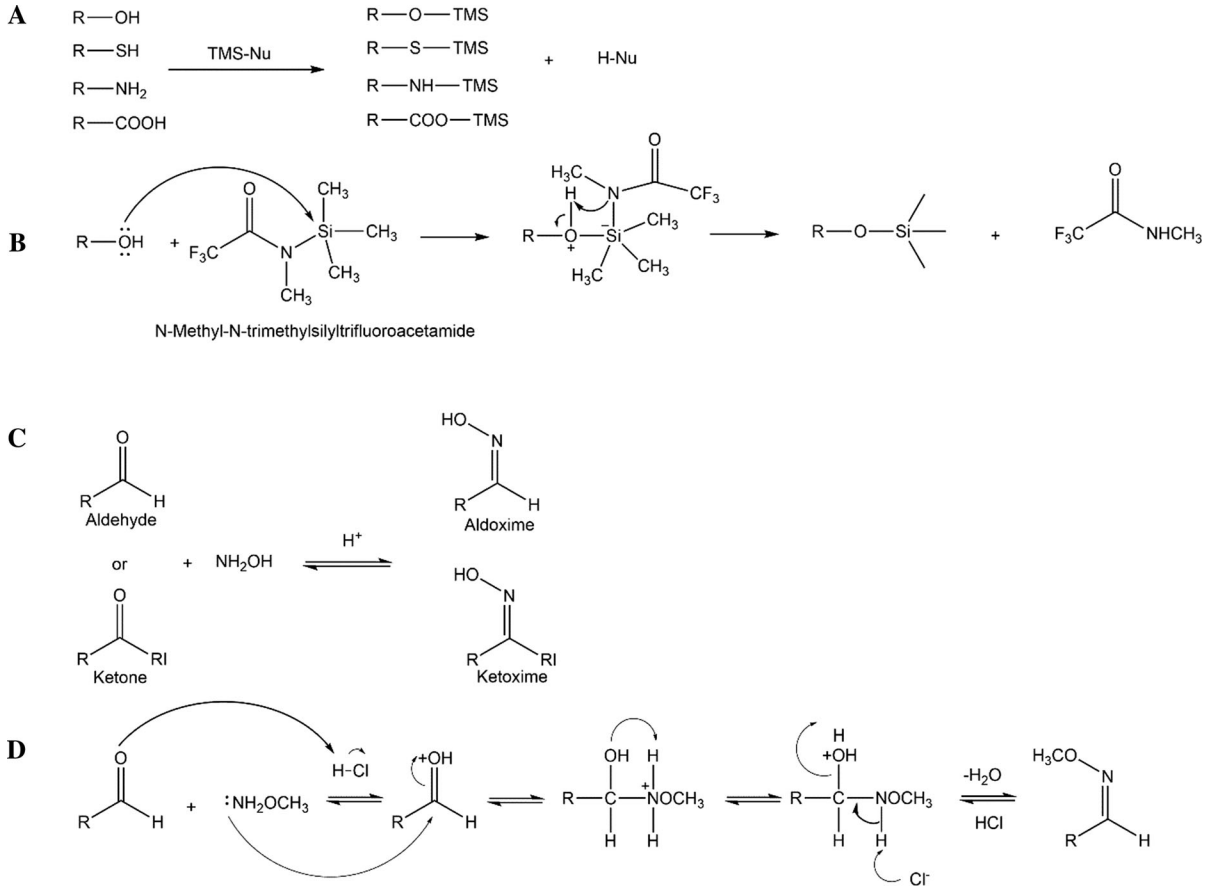

Fig. 3 Derivatization steps prior to GC–MS analysis: **a** Silylation reactions between hydrophilic groups and trimethylsilyl (TMS) reagent (Nu = nucleophile), **b** silylation reaction mechanism, **c** oximation reaction of aldehyde and ketone (e.g. open chain form of glucose and fructose), **d** reaction mechanism between methoxyamine hydrochloride and a generic aldehyde

These reactions are moisture sensitive therefore the sample must be completely dried. Thus, preparing MS samples is higher time-consuming compared to NMR, and more likely to generate unrepresentative samples. On the other hand, one of the most important advantage of using GC–MS compared to NMR is the very small amount of derivatised samples necessary for the analysis (typical injection volumes of 1 µl) to give high resolution spectra.

Salvia species

The genus *Salvia* consists of ca 900 species (Ayerza and Coates 2005) and its name comes from the latin word "salvere", referring to the curative properties of this plant. Nowadays, some species are still used all over the world for their nutritional properties and their beneficial effects on human health. Qualitative and quantitative differences described for the metabolites profile of the same genotype could be attributed to different technical issues, such as harvesting season, extraction methods, environmental factors, including altitude, climate and rainfall, abiotic or salt stress, and nutrient availability. Qualitative analysis by NMR spectroscopy has been obtained by 2D experiments, comparison of chemical shifts with standard compounds and with data reported in the literature. In Table 1 the typical identification peaks of the major metabolites from *Salvia* are listed. Peak integration and use of internal standards allowed respectively the relative and absolute quantification. Regarding MS identification, qualitative data were obtained by comparison with retention times and m/z values of

Table 1 Major metabolites from *Salvia* extracts and their typical identification peaks by ^{1}H NMR spectroscopy

Main compounds	Assignment	^{1}H (ppm range)[a]	Multiplicity [*J* (Hz)]
Aqueous extract			
Organic acids			
Acetic acid	CH_3	1.91–1.99	s
Citric acid	α,γ-CH	2.63	d [17.5]
	α^{I},γ^{I} –CH	2.79	d [17.5]
Malonic acid	CH_2	3.13–3.19	s
Lactic acid	CH_3	1.20–1.34	d [7.0]
Succinic acid	α,β-CH_2	2.41–2.52	s
Formic acid	HCOOH	8.35–8.48	s
Fumaric acid	α,β –CH=CH	6.49–6.52	s
Amino acids			
Alanine	β-CH_3	1.45–1.49	d [7.0]
Isoleucine	δ-CH_3	0.91–0.94	
	γ^{I}-CH_3	1.01–1.02	
Leucine	δ-CH_3	0.95–0.96	
Proline	γ-CH_2	2.00–2.08	m
Valine	γ^{I}-CH_3	0.99	d [7.0]
	γ-CH_3	1.01–1.04	d [7.0]
Carbohydrates			
α-Glucose	CH-1	5.24–5.37	d [4.0]
β-Glucose	CH-1	4.65–4.96	d [8.0]
Raffinose	Glc-C_1H	5.40–5.44	d [4.0]
Sucrose	Glc-C_1H	5.38–5.42	d [4.0]
Caffeoyl derivatives			
Caffeic acid	CH-8	6.34	d [16.0]
	CH-5	6.76	d [8.0]
	CH-6	6.94	dd [2.0, 8.0]
	CH-2	7.20	d [2.0]
	CH-7	7.50	d [16.0]
Chlorogenic acid	CH-5^{I}	6.95	d [8.0]
	CH-2^{I}	7.16	d [2.0]
Rosmarinic acid	CH-8	6.30–6.34	d [16.0]
	CH-7	7.51–7.58	d [16.0]
Organic phase			
Fatty acids	ω_1-CH_3	0.89	
	ω_3-CH_3	0.97	t [8.0]
	–$(CH_2)_n$–	1.28	
	γ-CH_2	1.61	
	allylic C	2.07	m
	β-CH_2	2.32	t [7.0]
	Diallylic C	2.81	
Glycerol	CH-1,3	4.22	
MUFA	CH=CH	5.28	
PUFA	CH=CH	5.37	

[a]ppm range is referred to several authors (de Falco et al. 2017b; Dai et al. 2010a; Jiang et al. 2014) and depends from the deuterated solvent used, pH of samples and instrument power

standard compounds available in laboratory and in data libraries. As for NMR, peak integration and use of internal standards allowed respectively the relative and absolute quantification to determine the metabolite profile of *Salvia*.

Salvia hispanica

Salvia hispanica L., also known as Chia, is a medicinal and dietary plant species used since ancient times by Mayan and Aztec. In the last years, researches have focused their attention especially on Chia dry indehiscent fruits, which are commonly called seeds (Bochicchio et al. 2015; Capitani et al. 2016; Jamboonsri et al. 2012). Chia seeds possess a very important role as functional food because of their chemical composition (de Falco et al. 2017a). Seeds are composed by total dietary fiber, contain up to 40% of oil with high content of unsaturated fatty acids, of which α-linolenic acid is the most abundant one (Ayerza and Coates 2005). Moreover, they are a good source of proteins, vitamins, minerals and antioxidants (de Falco et al. 2017a). There are very few scientific papers about metabolomics studies of Chia. In the most recent one, the metabolite profile of seven Chia (*S. hispanica* L.) seeds populations, three commercial (two black, and one white) and four early flowering genotypes (G3, G8, G17, W13.1, three black and one white, respectively), was investigated by NMR spectroscopy (de Falco et al. 2017b). The chemical composition of the different genotypes was investigated both from qualitative and quantitative point of view, through the identification of major metabolites by ^{1}H NMR analyses and the integration of NMR spectra followed by chemometrics tool. The research aimed at evaluating also the effect of agronomic management, such as fertilization with mineral nitrogen, on the metabolite composition of the commercial genotype. Several organic acids, free amino acids, carbohydrates, fatty acids and phenols were identified (Table 2) and results showed that apolar organic extracts were mainly composed of mono- and polyunsaturated fatty acids. The principal component analyses (PCA) plot separated the samples showing significant differences for signals related to carbohydrates, which were overall most abundant in commercial black genotype, while the analysis of seeds, cultivated at different nitrogen supply, showed a decrease in the content of carbohydrates and flavonoids, and an increase of aliphatic amino acids. The obtained data demonstrated that the ^{1}H NMR spectroscopy, followed by multivariate data analysis, can provide a detailed metabolite profile of biological samples, defining the main classes of organic compounds and selecting the best sample for food companies based on its nutraceuticals content. Amato et al. (2015) studied the metabolite profile of Chia leaves fertilized and not fertilized by using HPLC–ESI–MS. More recently de Falco et al. (2018a) investigated the yield and metabolic profile of the seeds of two chia populations, one commercial black (B) and one long-day flowering genotype (G8), in response to two irrigation levels. The analysis was performed by ultrasound assisted extraction (UAE) of seeds followed by gas chromatography–mass spectrometry (GC/MS) analysis. Differences in fatty acids and the major classes of organic compounds have been observed due to both genotype and irrigation, especially in the non-polar phase where irrigated samples showed a higher content of α-linolenic and other fatty acids and a lower oleic/linoleic ratio. Another recent study by de Falco et al. (2018b) analyzed for the first time the Chia metabolomics in response to mutation by the same technical approach. Mutants showed higher seed yield than commercial seeds and mutation affects chemical composition increasing nutraceutical properties. These results showed that metabolomics untargeted analysis is a very powerful methods to evaluate mutation but also to verify the effect of agronomic management such as fertilization and irrigation.

Salvia miltiorrhiza

S. miltiorrhiza Bunge, also known as red sage or Danshen, is a traditional Chinese medicinal herb used in the treatments of cardiovascular and liver diseases due to its antioxidant properties (Chen et al. 2000; Sugiyama et al. 2002; Wasser et al. 1998; Zhang et al. 1990). In particular, its dry roots are widely used in phytomedicine for the treatments of several diseases, such as atherosclerosis, myocardial ischemia and hepatic fibrosis, due to specific metabolites including salvianolic acids and the diterpenoids named tanshinones (Sieveking et al. 2005; Sugiyama et al. 2002). For example, tanshinones have been reported to possess antimicrobial activities against plant pathogens (Zhao et al. 2011), while tanshinone IIA exerts

Table 2 Metabolomics approach applied on different part of *Salvia hispanica*

Part of the plant	Analytical techniques	Data analysis	Metabolites	Quantity	References
Seeds	^{1}H-NMR	PCA	α-Glucose	63.2–73.3‰*	de Falco et al. (2017b)
Seeds	^{1}H-NMR	PCA	γ-Aminobutyrate	2.4–4.7‰*	de Falco et al. (2017b)
Leaves	HPLC–ESI–MS	ANOVA	Acetyl orientin	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Acetyl orientin derivative	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Acetyl vitexin	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Acetyl vitexin derivative	n.i.	Amato et al. (2015)
Seeds	^{1}H-NMR	PCA	Alanine	1.5–4.2‰*	de Falco et al. (2017b)
Leaves	HPLC–ESI–MS	ANOVA	Apigenin-7-*O*-glucoside	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Apigenin-C-pentose	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Apigenin-*O*-glucuronide	n.i.	Amato et al. (2015)
Seeds	^{1}H-NMR	PCA	Aspartate	1.9–2.9‰*	de Falco et al. (2017b)
Seeds	^{1}H-NMR	PCA	Caffeic acid	2.2–4.6‰*	de Falco et al. (2017b)
Leaves	HPLC–ESI–MS	ANOVA	Caffeic acid	n.i.	Amato et al. (2015)
Seeds	^{1}H-NMR	PCA	Chlorogenic acid	4–6.9‰*	de Falco et al. (2017b)
Leaves	HPLC–ESI–MS	ANOVA	Chlorogenic acid	n.i.	Amato et al. (2015)
Seeds	^{1}H-NMR	PCA	Citrate	3.3–7.4‰*	de Falco et al. (2017b)
Leaves	HPLC–ESI–MS	ANOVA	Coumaric acid-*O*-hexose	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Coumaroyl quinic acid-1	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Coumaroyl quinic acid-2	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Dihydrosinapic acid-*O*-hexose	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Dimethyl quercetin	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Ellagic acid-*O*-hexose	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Ferulic acid derivative	n.i.	Amato et al. (2015)
Seeds	^{1}H-NMR	PCA	Formate	0.1–4.2‰*	de Falco et al. (2017b)
Seeds	^{1}H-NMR	PCA	Fumarate	0.2–9.1‰*	de Falco et al. (2017b)
Seeds	^{1}H-NMR	PCA	Genistein	1.4–3.0‰*	de Falco et al. (2017b)
Seeds	^{1}H-NMR	PCA	Isoleucine	1.8–3.2‰*	de Falco et al. (2017b)
Leaves	HPLC–ESI–MS	ANOVA	Kaempferol-*O*-hexose desoxyhexose	n.i.	Amato et al. (2015)
Seeds	^{1}H-NMR	PCA	Lactate	0.5–1.7‰*	de Falco et al. (2017b)
Seeds	^{1}H-NMR	PCA	Leucine	0.7–1.2‰*	de Falco et al. (2017b)
Leaves	HPLC–ESI–MS	ANOVA	Luteolin-*O*-hexose	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Luteolin-7-*O*-glucoside	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Luteolin-*O*-glucuronide	n.i.	Amato et al. (2015)

Table 2 continued

Part of the plant	Analytical techniques	Data analysis	Metabolites	Quantity	References
Seeds	^{1}H-NMR	PCA	Malonate	0.7–2‰*	de Falco et al. (2017b)
Seeds	^{1}H-NMR	PCA	*N*-Acetylglutamate	2.4–4.8‰*	de Falco et al. (2017b)
Leaves	HPLC–ESI–MS	ANOVA	Naringenin	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Orientin (luteolin-6-C-glucoside)	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Orientin derivative	n.i.	Amato et al. (2015)
Seeds	^{1}H-NMR	PCA	Proline	1.8–3.7‰*	de Falco et al. (2017b)
Seeds	^{1}H-NMR	PCA	Quercetin	1.2–2.7‰*	de Falco et al. (2017b)
Leaves	HPLC–ESI–MS	ANOVA	Quercetin methyl ether	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Quercetin-*O*-hexose desoxyhexose	n.i.	Amato et al. (2015)
Seeds	^{1}H-NMR	PCA	Raffinose	82.4–103.8‰*	de Falco et al. (2017b)
Seeds	^{1}H-NMR	PCA	Rosmarinic acid	9.2–14‰*	de Falco et al. (2017b)
Leaves	HPLC–ESI–MS	ANOVA	Rosmarinic acid	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Salvianolic acid F isomer	n.i.	Amato et al. (2015)
Seeds	^{1}H-NMR	PCA	Succinate	1.6–3.8‰*	de Falco et al. (2017b)
Seeds	^{1}H-NMR	PCA	Sucrose	59.5–78.5‰*	de Falco et al. (2017b)
Seeds	^{1}H-NMR	PCA	Valine	2.7–4.5‰*	de Falco et al. (2017b)
Leaves	HPLC–ESI–MS	ANOVA	Vitexin (apigenin-8-C glucoside)	n.i.	Amato et al. (2015)
Leaves	HPLC–ESI–MS	ANOVA	Vitexin derivative	n.i.	Amato et al. (2015)

n.i. not indicated

*Range metabolite contents in *S. hispanica* extracts from different genotypes

vasorelaxative, antiarrhythmic and anti-cancer activities (Gao et al. 2008; Gong et al. 2010; Sun et al. 2008). Primary metabolites such as carbohydrates, amino acids and organic acids are essential for plant growth and maintenance of cells. However, liquid chromatography coupled with mass spectrometry (LC–MS) methods are more suitable for secondary metabolites such as polyphenolic acids and diterpenoids. To establish a method for detecting in a one-step procedure both primary and secondary metabolites in *S. miltiorrhiza*, Jiang et al. (2014) used ^{1}H-NMR fingerprint coupled with sparse partial-least-squares discriminant analysis (sPLS-DA). Metabolites were quantified and their levels evaluated by Kruskal–Wallis tests. In this study, *S. miltiorrhiza* root extracts were collected from four different regions of China (Zhongjiang, Linqu, Bozhou and Anguo). Twenty-six primary and secondary metabolites were identified and metabolomics studies suggest that malonate and succinate can be possibly recognised as the key markers for discriminating the geographical origin of *S. miltiorrhiza* roots. Dai et al. (2010a) analyzed the metabolite compositions of three cultivars of *S. miltiorrhiza* Bunge roots and a cultivar obtained from four different growing locations using NMR coupled to LC–DAD–MS methods to assess the effectiveness of these combined approaches in plant metabolomics. They also acquired ^{1}H–^{1}H COSY, ^{1}H–^{1}H TOCSY, ^{1}H J-resolved, ^{1}H–^{13}C HSQC and ^{1}H–^{13}C HMBC 2D NMR spectra on selected samples for resonance assignment purposes. The results showed that the metabolome was dominated by 4 polyphenolic acids

Table 3 Different analytical techniques and data analyses on metabolomics approach of *Salvia miltiorrhiza* roots

Analytical techniques	Data analysis	Secondary metabolites	Quantity	References
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	1,2-Dihydro-tanshinone I	n.r.	Dai et al. (2010a, b)
LC–qTOF–MS	PLS-DA	12-Deoxy-6,7 dehydroroyleanone*	10.4 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	15,16-Dihydro-tanshinone I	n.r.	Dai et al. (2010a, b)
HPLC–MS	PCA and OPLS-DA	15,16-Dihydro-tanshinone I	0.13–1.14 g/kg[a2]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	15,16-Dihydro-tanshinone I	0.94–2.19 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	15,16-Dihydro-tanshinone I	0.14–0.45 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	15,16-Dihydro-tanshinone I	0.07–0.37 g/kg[e]	Zhao et al. (2016)
LC–qTOF–MS	PLS-DA	16-Hydroxy-6,7-didehydroferruginol*	7.9 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	1-Ketoiso-crypto-tanshinone	n.r.	Dai et al. (2010a, b)
LC–qTOF–MS	PLS-DA	1*R*-Hydroxymiltirone*	1357.3 μg/g FW	Cui et al. (2015)
^{1}H-NMR	PCA and sPLS-DA	2-Hydroxy-3-methyl valerate	n.r.	Jiang et al. (2014)
^{1}H-NMR	PCA and sPLS-DA	3-Hydroxy-3-methyl glutarate	n.r.	Jiang et al. (2014)
LC–qTOF–MS	PLS-DA	3-Hydroxy-cryptotanshinone	10.0 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	3-Hydroxysalvilenone	7.9 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	3α,24-Dihydro-xyolean-12-en-28-oic acid	n.r.	Dai et al. (2010a, b)
LC–qTOF–MS	PLS-DA	3α-Hydroxymethylene-tanshinquinone	204.1 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	4-Methylenemiltirone	246.8 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	5,6-Dehydrosugiol	8.0 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	7-Hydroxy-12-methoxy-20-nor-abieta-1,5(10),7,9,12-pentaen-6,14-dione*	13.5 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	α-Galactose	n.r.	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	α-Glucose	n.r.	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	β-Glucose	n.r.	Dai et al. (2010a)
^{1}H-NMR	PCA and sPLS-DA	β-Glucose	4.73–8.65 mg/ml[a3]	Jiang et al. (2014)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	β-Sitosterol	n.r.	Dai et al. (2010a)
^{1}H-NMR	PCA and sPLS-DA	γ-Aminobutyrate	4.93–11.55 mg/ml[a3]	Jiang et al. (2014)

Table 3 continued

Analytical techniques	Data analysis	Secondary metabolites	Quantity	References
LC–qTOF–MS	PLS-DA	7β-Hydroxy-8,13abieta-diene-11,12-dione*	92.1 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	*N*-Acetylglutamate	0–5.28 mg/g[a1]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	*N*-Acetylglutamate	4.0–5.28 mg/g[b]	Dai et al. (2010a)
GC–QqQ–MS	PLS-DA	Abietatriene	0.6 μg/g DW	Cui et al. (2015)
^{1}H-NMR	PCA and sPLS-DA	Acetate	1.38–2.88 mg/ml[a3]	Jiang et al. (2014)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Alanine	0.36–1.01 mg/g[a1]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Alanine	0.76–0.94 mg/g[c]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Alanine	1.46–1.80 mg/g[d]	Dai et al. (2010b)
^{1}H-NMR	PCA and sPLS-DA	Benzoate	2.30–7.82 mg/ml[a3]	Jiang et al. (2014)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Caffeic acid	n.r.	Dai et al. (2010a)
LC–qTOF–MS	PLS-DA	Cryptanol*	11.17 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	Crypto-japonol*	7.8 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Crypto-tanshinone	n.r.	Dai et al. (2010b)
^{1}H-NMR	PCA and sPLS-DA	Crypto-tanshinone	n.r.	Jiang et al. (2014)
HPLC–MS	PCA and OPLS-DA	Crypto-tanshinone	0.17–3.65 g/kg[a2]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Crypto-tanshinone	3.65–7.95 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Crypto-tanshinone	0.59–1.41 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Crypto-tanshinone	0.16–1.48 g/kg[e]	Zhao et al. (2016)
LC–qTOF–MS	PLS-DA	Crypto-tanshinone	15,959.6 μg/g FW	Cui et al. (2015)
GC–QqQ–MS	PLS-DA	Crypto-tanshinone	6.8 μg/g DW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Danshensu	n.r.–6.29 mg/g[a]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Danshensu	7.11–7.46 mg/g[b]	Dai et al. (2010a)
HPLC–MS	PCA and OPLS-DA	Danshensu	0.25–0.33 g/kg[a2]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Danshensu	0.14–0.25 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Danshensu	0.15–0.18 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Danshensu	0.14–0.33 g/kg[e]	Zhao et al. (2016)
GC–QqQ–MS	PLS-DA	Danshenxinkun B	191.9 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	Dihydrotanshinone I	1233.6 μg/g FW	Cui et al. (2015)
^{1}H-NMR	PCA and sPLS-DA	Dihydrotanshinone I	n.r.	Jiang et al. (2014)

Table 3 continued

Analytical techniques	Data analysis	Secondary metabolites	Quantity	References
GC–QqQ–MS	PLS-DA	Ferruginol	29.5 μg/g DW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Ferulic acid	n.r.	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Glutamine	4.86–45.23 mg/g[a1]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Glutamine	4.0–9.21 mg/g[b]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Glutamine	27.71–47.28 mg/g[c]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Glutamine	14.45–1.30 mg/g[d]	Dai et al. (2010b)
^{1}H-NMR	PCA and sPLS-DA	Histidine	0.38–1.88 mg/ml[a3]	Jiang et al. (2014)
LC–qTOF–MS	PLS-DA	Hydroxytanshinone IIA	200.4 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Isoleucine	0.37–0.95 mg/g[a1]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Isoleucine	0.94–1.06 mg/g[c]	Dai et al. (2010b)
LC–qTOF–MS	PLS-DA	Isotanshinone IIA	107.1 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Lactate	0.48–0.95 mg/g[a1]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Lactate	1.18–1.30 mg/g[c]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Lactate	1.14–2.43 mg/g[d]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Lithospermic acid	4.12–9.49 mg/g[a]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Lithospermic acid	8.07–10.41 mg/g[b]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Lithospermic acid	3.17–8.70 mg/g[c]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Lithospermic acid	4.45–5.03 mg/g[d]	Dai et al. (2010b)
HPLC–MS	PCA and OPLS-DA	Lithospermic acid	1.44–2.96 g/kg[a2]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Lithospermic acid	1.71–2.82 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Lithospermic acid	1.11–1.73 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Lithospermic acid	0.95–2.96 g/kg[e]	Zhao et al. (2016)

Table 3 continued

Analytical techniques	Data analysis	Secondary metabolites	Quantity	References
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Malate	9.79–23.99 mg/g[a1]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Malate	6.62–8.96 mg/g[b]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Malate	12.43–15.39 mg/g[c]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Malate	26.10–26.30 mg/g[d]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Malonate	n.r.	Dai et al. (2010a)
^{1}H-NMR	PCA and sPLS-DA	Malonate	1.61–6.80 mg/ml[a3]	Jiang et al. (2014)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Maslinic acid	n.r.	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Melibiose	n.r.	Dai et al. (2010a)
^{1}H-NMR	PCA and sPLS-DA	Melibiose	1.65–5.37 mg/ml[a3]	Jiang et al. (2014)
LC–qTOF–MS	PLS-DA	Methyltanshinonate	2137.8 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Miltipolone	n.r.	Dai et al. (2010a)
GC–QqQ–MS	PLS-DA	Miltiradiene	0.2 μg/g DW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Miltirone	n.r.	Dai et al. (2010a)
LC–qTOF–MS	PLS-DA	Miltirone	6271.2 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	Neocryptotanshinone	7.9 μg/g FW	Cui et al. (2015)
GC–QqQ–MS	PLS-DA	Neophytadiene	0.03 μg/g DW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	Pomiferin G*	12.0 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	Prionoid E*	105.8 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	Prineoparaquinone*	41.6 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Proline	n.r.	Dai et al. (2010a)
^{1}H-NMR	PCA and sPLS-DA	Proline	6.31–9.05 mg/ml[a3]	Jiang et al. (2014)
LC–qTOF–MS	PLS-DA	Przewalskin	11.3 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	Przewalskin y-1	157.8 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Retusin	n.r.	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Rosmarinic acid	2.80–5.18 mg/g[a1]	Dai et al. (2010a)

Table 3 continued

Analytical techniques	Data analysis	Secondary metabolites	Quantity	References
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Rosmarinic acid	2.82–3.22 mg/g[b]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Rosmarinic acid	1.80–7.06 mg/g[c]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Rosmarinic acid	4.19–4.66 mg/g[d]	Dai et al. (2010b)
HPLC–MS	PCA and OPLS-DA	Rosmarinic acid	2.38–5.34 g/kg[a2]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Rosmarinic acid	1.90–3.98 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Rosmarinic acid	1.51–2.51 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Rosmarinic acid	1.22–5.34 g/kg[e]	Zhao et al. (2016)
^{1}H-NMR	PCA and sPLS-DA	Rosmarinic acid	n.r.	Jiang et al. (2014)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Raffinose	316.03–426.63 mg/g[a1]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Raffinose	351.41–495.55 mg/g[b]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Raffinose	321.13–436.92 mg/g[c]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Raffinose	290.67–311.14 mg/g[d]	Dai et al. (2010b)
^{1}H-NMR	PCA and sPLS-DA	Raffinose	20.11–72.81 mg/ml[a3]	Jiang et al. (2014)
^{1}H-NMR	PCA and sPLS-DA	Salvianic acid	3.16–9.34 mg/ml[a3]	Jiang et al. (2014)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Salvianolic acid B	68.07–134.23 mg/g[a1]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Salvianolic acid B	58.79–65.01 mg/g[b]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Salvianolic acid B	28.68–130.0 mg/g[c]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Salvianolic acid B	81.79–87.08 mg/g[d]	Dai et al. (2010b)
^{1}H-NMR	PCA and sPLS-DA	Salvianolic acid B	11.03–29.91 mg/ml[a3]	Jiang et al. (2014)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Salvianolic acid F	n.r.	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Salvianolic acid H/I	n.r.	Dai et al. (2010a)
HPLC–MS	PCA and OPLS-DA	Salvianolic acid H/I	39.2349.13 g/kg[a2]	Zhao et al. (2016)

Table 3 continued

Analytical techniques	Data analysis	Secondary metabolites	Quantity	References
HPLC–MS	PCA and OPLS-DA	Salvianolic acid H/I	39.23–54.08 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Salvianolic acid H/I	35.36–48.61 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Salvianolic acid H/I	23.82–49.13 g/kg[e]	Zhao et al. (2016)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Salvianolic acid l	n.r.	Dai et al. (2010a)
LC–qTOF–MS	PLS-DA	Salvisyrianone*	24.5 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	Saprorthoquinone*	101.2 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Succinate	1.54–3.54 mg/g[a1]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Succinate	2.02–2.47 mg/g[b]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Succinate	1.68–3.05 mg/g[c]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Succinate	4.96–6.04 mg/g[d]	Dai et al. (2010b)
^{1}H-NMR	PCA and sPLS-DA	Succinate	2.20–5.10 mg/ml[a3]	Jiang et al. (2014)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Sucrose	9.98–80.25 mg/g[a1]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Sucrose	7.49–18.01 mg/g[b]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Sucrose	23.98–75.18 mg/g[c]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Sucrose	17.77–21.25 mg/g[d]	Dai et al. (2010b)
^{1}H-NMR	PCA and sPLS-DA	Sucrose	10.20–34.13 mg/ml[a3]	Jiang et al. (2014)
LC–qTOF–MS	PLS-DA	Sugiol	59.3 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Tanshinaldehyde	n.r.	Dai et al. (2010a)
LC–qTOF–MS	PLS-DA	Tanshindiol A	47.3 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	Tanshindiol B	37.4 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	Tanshindiol C	270.3 μg/g FW	Cui et al. (2015)
LC–qTOF–MS	PLS-DA	Tanshinol B	768.0 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Tanshinone I	n.r.	Dai et al. (2010a)
^{1}H-NMR	PCA and sPLS-DA	Tanshinone I	n.r.	Jiang et al. (2014)
HPLC–MS	PCA and OPLS-DA	Tanshinone I	0.25–1.79 g/kg[a2]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Tanshinone I	1.79–3.62 g/kg[e]	Zhao et al. (2016)

Table 3 continued

Analytical techniques	Data analysis	Secondary metabolites	Quantity	References
HPLC–MS	PCA and OPLS-DA	Tanshinone I	0.37–0.89 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Tanshinone I	0.25–0.70 g/kg[e]	Zhao et al. (2016)
LC–qTOF–MS	PLS-DA	Tanshinone I	2252.3 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Tanshinone IIA	n.r.	Dai et al. (2010a)
HPLC–MS	PCA and OPLS-DA	Tanshinone IIA	0.19–3.94 g/kg[a2]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Tanshinone IIA	3.94–6.65 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Tanshinone IIA	0.88–2.02 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Tanshinone IIA	0.19–2.13 g/kg[e]	Zhao et al. (2016)
HPLC–MS	PCA and OPLS-DA	Tanshinone IIA	19,520.4 μg/g FW	Cui et al. (2015)
^{1}H-NMR	PCA and sPLS-DA	Tanshinone IIA	15.93–24.60 mg/ml[a3]	Jiang et al. (2014)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Tanshinone IIB	n.r.	Dai et al. (2010a)
LC–qTOF–MS	PLS-DA	Tanshinone IIB	2088.3 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Tormentic acid	n.r.	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Trijuganone B	n.r.	Dai et al. (2010a)
LC–qTOF–MS	PLS-DA	Trijuganone B	6957.5 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Trijuganone C	n.r.	Dai et al. (2010a)
LC–qTOF–MS	PLS-DA	Trijuganone C	142.4 μg/g FW	Cui et al. (2015)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Valine	0.46–1.35 mg/g[a1]	Dai et al. (2010a)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Valine	1.20–1.46 mg/g[c]	Dai et al. (2010b)
NMR and HPLC–DAD–ESI–MS	PCA and OPLS-DA	Yunnaneic acid D	n.r.	Dai et al. (2010a)

n.r. not reported because of signal weakness or overlapping, *FW* fresh weight, *DW* dry weight

*Compounds not isolated in *S. miltiorrhiza* before

Range metabolite contents in *S. miltiorrhiza* extracts from different

[a]Geographic locations: [1]Sichuan, Hubei, Hebei, and Henan; [2]Zhuyang, Changqing and Taian; [3]Zhongjiang, Linqu, Bozhou and Anguo

[b]Cultivars: *Sativa*, *Foliolum* and *Silcestris*

[c]Drying processes: freeze-drying, sun-drying and air-drying

[d]Solvents: boiling water, 50% aqueous ethanol, 50% aqueous methanol and chloroform–methanol mixture (3:1)

[e]Genotypes

including salvianolic acid B, lithospermic acid, rosmarinic acid and danshensu along with 28 primary metabolites including 5 sugars, 8 carboxylic acids, 10 amino acids and choline, while *N*-acetylglutamate, aspartate and fumarate were detected for the first time in this plant (Table 3). Moreover, their finding showed differences for samples from different locations and between three ecotypes in both NMR and LC–MS spectra. These results indicated that a combined approach of NMR and LC–MS provides different but complementary information. In particular, NMR methods were effective to quantitatively detect both primary and secondary metabolites, whereas LC–DAD–MS methods were excellent for the detection of minor secondary metabolites. They demonstrated that these combined methods provide an excellent approach for targeted analysis of plant secondary metabolite compositions associated with growing environments and ecotypic cultivars. In another paper, Dai et al. (2010b) demonstrated by using the same combined methods how the metabolite profile of *S.miltiorrhiza* roots changes with water depletion, different drying processes, and different extraction solvents. The fresh roots were subjected to freeze-drying after snap-frozen in liquid nitrogen, sun-drying and air-drying, while to investigate the effects of extraction solvents on the salvia roots metabolites, the same dried raw materials were extracted ultrasonically with boiling water (solvent A), 50% aqueous ethanol (solvent B), 50% aqueous methanol (solvent C) and chloroform–methanol mixture (3:1, v/v) (solvent D), respectively. The PCA scores plot of NMR data showed that, compared with extracts from solvent A,B and C, chloroform–methanol extracts contained high amounts of the diterpenoids tanshinone I, 15,16-dihydrotanshinone I, 1,2-dihydrotanshinone I and cryptotanshinone but less amounts of sugars, amino acids and salvianolic acid B. Furthermore, the raffinose levels increased during the sun-drying process but decreased during the air-drying process. The sun-dried samples contained significant lower levels of polyphenolic acids (e.g., rosmarinic acid and salvianolic acid B) and tanshinones than the air-dried ones. These findings strongly suggest the importance of metabolisms in plant adaptation to osmotic stress. Zhao et al. (2016) also studied the metabolite composition of different genotypes of *S. miltiorrhiza* roots, growing in different geographical area of China (Zhuyang, Changqing and Taian). They demonstrated that LC/MS-based metabolomics is a holistic and effective method to monitor variations of the bioactive compounds, showing how the contents of phenolic acids and tanshinones were affected by locations as well as genotypes. Specifically, genotypes grown in Zhuyang as compared to those grown in Changqing and Taian had higher phenolic acids and tanshinones content (Table 3). Another work on *S. miltiorrhiza* roots was conducted by Cui et al. (2015) who reported the characterization of the enzymatic families in tanshinone production in roots versus aerial tissues. Investigation of gene structure, positive selection, molecular docking, and mutational analysis were performed to clarify the functional diversity of diterpene synthases, which play a very important role in diterpenoid biosynthesis. Moreover, they provide a metabolomics approach with two independent analytical methods for the detection of all metabolites of two extracts: UHPLC–qTOF–MS and GC–QqQ–MS analysis for methanol and hexane extracts, respectively (Cui et al. 2015). GC–QqQ–MS analysis was used for the identification of metabolites not detectable with the UHPLC–qTOF–MS method, because of their hydrophobicity (Table 3). The UHPLC–ESI–qTOF–MS methods revealing 39 metabolites, while GC–EI–QqQ–MS 19. After identification and quantification, statistical analyses were performed with PLS-DA.

Salvia officinalis

S. officinalis is traditionally used as culinary herb, in beverages, as herbal product in the form of herbal teas, or extracts, in cosmetics and perfumery. Its composition and biological activities have been the subject of previous reports (Baricevic and Bartol 2000; El-Feky and Aboulthana 2016). In particular, this plant is rich in essential oils, with main constituents α- and β-thujone, camphor, 1,8-cineole and borneol. (Baydar et al. 2013; Seidler-Lozykowska et al. 2015). *S. officinalis* is also rich in polyphenolic compounds, including rosmarinic acid, carnosic acid, carnosol, chlorogenic and salvianolic acids (Tounekti and Munné-Bosch 2012; El-Feky and Aboulthana 2016). Sarrou et al. (2017) established a reliable tools to characterize the genomic and metabolic diversity in *S. officinalis* leaves grown in Greece. Based on the difference at the genomic level and in metabolic fingerprints, they used as selecting criteria the high polyphenol content and the essential oil composition

Table 4 Metabolomics approach on *Salvia officinalis* leaves by UPLC–MS/MS and PCA (phenols) and GC–MS and PCA (volatile compounds and oil content) taken by Sarrou et al. (2017)

Volatile compounds	Quantity (%)[a]	Phenols	Quantity (mg/g)[a]
4-Terpineol	0.2–0.4	1,5-Dicaffeoylquinic acid	0–0.035
Alloaromadrenene	0–0.2	2,4-Dihydroxy-benzoic acid	0.002–0.006
a-Pinene	3.4–8.4	2,5-Dihydroxy-benzoic acid	0.003–0.01
a-Terpinene	0.2–0.4	3,5-Dihydroxy-benzoic acid	0.006–0.009
α-Copaene	0–0.2	Acetovanillone	0.009–0.033
α-Humulene	2.2–9.9	Apigenin	0.012–0.025
α-Terpineol	0.1–0.4	Apigenin-7-*O*-glucoside	0.268–0.610
α-Terpinolene	0.1–0.8	Caffeic acid	0.145–0.204
α-Thujone	10.5–29	Carnosol	0.102–1.159
Bicyclogermacrene	0.3–0.7	Chlorogenic acid	0.001–0.073
Borneol	2–6.2	Coniferyl alcohol	0.008–0.011
Bornyl acetate	0–3	Cryptochlorogenic acid	0–0.002
β-Caryophyllene	0.8–9	Daphnetin	0.008–0.015
β-Pinene	2.2–5	Esculin	0.005–0.01
β-Thujone	1.2–3.3	Ferulic acid	0.022–0.032
Camphene	2.3–5.5	Hesperidin	0.026–0.131
Camphor	4.5–12.5	Luteolin	0.179–0.249
Caryophyllene oxide	0.1–1	Luteolin-7-*O*-gluccoside	0.176–0.465
Cineol	10.8–24.4	Naringenin	0.001–0.002
cis-*β*-Ocimene	0.7–2.3	*p*-Hydroxybenzoic acid	0.009–0.022
γ-cadinene	0–0.1	Protocatechuic acid	0.004–0.009
γ-muurolene	0.1–1.3		
γ-Terpinene	0.4–0.7		
δ-Cadinene	0–0.4		
δ-Terpineol	0–0.3		
Humulene epoxide	0.4–1.3		
Limonene	1.1–1.8		
Linalool	0–0.5		
Manool	1.9–3.6		
Myrcene	0.7–0.9		
p-Cymene	0.3–0.6		
Salvene *E*	0–0.2		
Salvene *Z*	0.4–1.1		
tr-Pinocamphone	0–0.5		
tr-*β*-Ocimene	0.1–0.5		
Viridiflorol	2.8–12.4		

[a]Range metabolite contents in *S. officinalis* extracts from different geographic locations in Greece

in the diversification of several populations of *S. officinalis*. Metabolomics analyses of hydrophilic extract were performed with a targeted UPLC–MS/MS method, while the essential oil contents were identified using GC–MS. They found rosmarinic acid, carnosol, syringic acid, caffeic acid, hesperidin, apigenin-7-*O*-glucoside, luteolin, luteolin-7-*O*-glucoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside as well as carnosic acid as the main compounds and their concentration varied significantly (Table 4) among the seven genotypes. These finding shows the efficiency of metabolite profiling using LC–MS/MS and GC–MS to characterize the quality of medicinal and aromatic plants. Metabolomics approach is very useful not only to study the same genotype with several treatments but also to select and characterize extracts from different plants. For example, Brahmi et al. (2015) compared the hydroalcoholic

extracts of eleven Algerian medicinal plants (*Aloysia triphylla, Apium graveolens, Coriandrum sativum, Laurus nobilis, Lavandula officinalis, Marrubium vulgare, Mentha spicata, Inula viscosa, Petroselinum crispum, Salvia officinalis,* and *Thymus vulgaris*) through ^{1}H NMR spectroscopy. Whereas, Mattoli et al. (2006) determined the metabolic profile of eight different plants (*Cimicifuga racemosa, Filipendula vulgaris, Helichrysum italicum, Spiraea ulmaria, Filipendula ulmaria, Salvia officinalis, Helianthus annuus, Achillea millefolium*) obtained through ESI–MS and NMR spectroscopy. They found that the positive ion ESI spectra of the extracts were highly complicated in all cases, in contrast, the negative ion ESI mass spectra were much better defined. In the PCA plot different samples of the same species appear well clustered and separated from the other species, while the aromatic region of NMR spectra were less useful for the detection of clusters among the different species. For this reason, they selected the ESI–MS method, particularly with negative ion mode, as reliable metabolomics fingerprint for a group of plants.

Other species

The untargeted metabolomics approach provided reliable and objective techniques in the chemotaxonomic evaluation of several *Salvia* species. For example, Sandasi et al. (2012) compared two species of *Salvia* (*S. runcinata* and *S. stenophylla*) by using mid-infrared spectroscopy (MIRS) and GC–MS methods. Results showed that MIRS produced better clustering patterns compared to GC–MS. Moreover, *S. runcinata* showed greater intra-species chemical variation compared to *S. stenophylla* using both methods.

Data analysis

The untargeted metabolomics data, obtained by the identification and quantification of as many metabolites as possible, is subsequently statistically processed. Statistical approaches require replicates of samples preparation. Usually, multivariate data analysis (MVDA) techniques are used to maximize classification of samples. Principal components analysis (PCA), partial least square (PLS), hierarchical cluster analysis (HCA) and self-organizing mapping (SOM) have been extensively employed as statistical tools for metabolomics. PCA is a useful approach to reduce the dimensionality of a large data set and is the most used chemometric tool as clustering technique. PCA describes the variance between original variables (in this case metabolites concentrations) through a linear combination of new variables, which are principal components (PC), identifying how samples are different from each other and which variables (metabolites concentrations) contribute most to this difference. On the contrary, PLS regression is more used to create a prediction model in metabolomics studies (Tarachiwin et al. 2008). In general, PLS is used to show the information contained in two data sets (X = predictor matrix, Y = response matrix), while PLS discriminant analysis (PLS-DA) is a classic PLS regression where the response variables are qualitative (Y = categories of the samples). Sparse partial-least-squares discriminant analysis (sPLS-DA) allows variable selection in a one-step procedure, improving interpretability via valuable graphical outputs (Jiang et al. 2014). HCA is also used frequently in metabolomics and measures the distance between rows or columns of a data matrix. This approach can be employed with an agglomerative or a divisive method and data are presented in a diagram known as dendrogram. SOM is a noncluster method widely used for genomics and transcriptomics (Abe et al. 2003; Hirai et al. 2004), but more recently it was also applied to monitor metabolic dynamics in rice leaves (Sato et al. 2008).

Conclusion

This review presents the metabolomics approaches applied to different species of *Salvia*. Within several techniques, NMR spectroscopy and mass spectrometry analysis were the most employed by researchers. For this reason, the major advantages and limitations of these analytical tools were discussed in details. In conclusion, mass spectrometry, particularly GC–MS, has a detection threshold of 10^{-12} mol, which is more sensitive than ^{1}H NMR spectroscopy where a value of 10^{-6} mol is observed. In both analytical methods, extraction step is required, but all classes of compounds must be derivatized in GC–MS analysis, which takes additional time, processing, and variance in

comparison to the NMR tool. Although NMR spectroscopy is a non-destructive technique, it is not the most suitable for the identification of lipids and volatile compounds, for which MS provides better results. Therefore, a single analytical method will not provide the best result for metabolomics analyses and a combined approach, such as parallel MS and NMR analyses on the same sample, would be an ideal way to increase the detection of different classes of metabolites.

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