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Extraction for Metabolomics: Access to The Metabolome

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ABSTRACT:
Introduction – The value of information obtained from a metabolomic study depends on how much of the metabolome is present in analysed samples. Thus, only a comprehensive and reproducible extraction method will provide reliable data because the metabolites that will be measured are those that were extracted and all conclusions will be built around this information.
Objective – To discuss the efficiency and reliability of available sample pre-treatment methods and their application in different fields of metabolomics.
Methods – The review has three sections: the first deals with pre-extraction techniques, the second discusses the choice of extraction solvents and their main features and the third includes a brief description of the most used extraction techniques: microwave-assisted extraction, solid-phase extraction, supercritical fluid extraction, Soxhlet and a new method developed in our laboratory – the comprehensive extraction method.
Results – Examination of over 200 studies showed that sample collection, homogenisation, grinding and storage could affect the yield and reproducibility of results. They also revealed that apart from the solvent used for extraction, the extraction techniques have a decisive role on the metabolites available for analysis.
Conclusion – It is essential to evaluate efficacy and reproducibility of sample pre-treatment as a first step to ensure the reliability of a metabolomic study. Among the reviewed methods, the comprehensive extraction method appears to provide a promising approach for extracting diverse types of metabolites.

Keywords: Extraction; sample pre-treatment; metabolomics

Introduction

With advances in technology and the ability to collect and process enormous amounts of data, the advance of life sciences has seen a change from a reductionist approach towards that provided by systems biology, that is, measuring biological aspects of a whole system and its interaction with its surroundings, rather than targeting one single part of it. In today's research world, omics techniques such as proteomics, transcriptomics, genomics and metabolomics have become an integral part of systems biology (Mushtaq *et al*., 2013).

In the past 10 years, metabolomics has developed into an important tool for all kinds of applications, including studies on diseases, toxicity, quality control and natural products (Verpoorte *et al*., 2007). The ultimate goal of metabolomics is the chemical characterisation of a phenotype through the qualitative and quantitative analysis of all metabolites in an organism and the measurement of the change in the metabolite profile due to some type of challenge or perturbation (Oliver *et al*., 1998). A metabolomics experiment generally consists of the following steps: sample collection, extraction, analysis of the extract, data reduction and statistics. The results depend heavily upon the very initial steps that involve sample collection and extraction. Researchers usually collect samples appropriately but often ignore the importance of the extraction step, applying protocols based on their experience but omit to validate, or at least perform some evaluation of, their efficiency. Generally, the idea of metabolomics is that the samples to be analysed contain all the intact metabolites that can be extracted using different solvents or techniques from an organism. Thus, any non-extracted metabolites will greatly affect the overall quality of the metabolomic study, which will be based on incomplete information. Thus, it is vital to pay much attention to the extraction protocol design. Prasad and Ferenci (2003) summarised this idea, defining an efficient extraction protocol as one that should extract the largest number of metabolites and be non-destructive. For this, it is important to remember that the extraction mechanism must not only consider solute–solvent interactions but also the dissociation of the solute or analyte from the matrix. It means that along with proper solvent selection, both pre-extraction techniques and the treatment of the matrix during extraction play an important part in the release of metabolites, and to achieve good results not only should the type of solvent used be taken into account but also the physico-chemical characteristics of the matrix, effect of the pH on the matrix, contact time and compartmentalisation of metabolites (Silas and Villas, 2006).

The main objective of this review is to highlight the importance of extraction in the field of metabolomics and to discuss how different kinds of extraction techniques and conditions

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can affect the outcome of the study. We will try to cover the pre-treatment of samples before extraction and the types of extraction techniques available to date.

Sample preparation

Sample collection and quenching

Sampling for a metabolomics analysis should be fast because of the high rate of metabolome turnover in a living organism (Bolten *et al*., 2007). Immediately after sampling, however, the metabolites themselves might suffer changes, so that considering both factors, it is clearly very important to collect samples fast and take steps to stop any kind of biochemical activity right away. It is also essential to keep the relevance of the sampling time in mind because diurnal patterns can cause changes in the metabolic profile as well as gene regulation patterns (Espinoza *et al*., 2010). For example, it is now a well-known fact that in plants, the malic acid and sugars content fluctuates during the daily cycle (Queiroz, 1974). Similarly, Urbanczyk-Wochniak *et al*. (2005) reported the fluctuation of both primary and secondary metabolites in potatoes throughout the daily cycle. This should also be considered for microorganism or animal sampling. Another important issue is the homogeneity and tissue specificity of samples. Bando *et al*. (2010) concluded that in the case of urine and plasma samples of mice, the pooling time, temperature of tubes used for collecting the urine, or anti-coagulant agents used for blood plasma and sampling sites of blood (e.g. jugular vein or abdomen) could cause a significant variation in results. In the case of plants, the metabolomic profiles of samples collected at different stages of development were found to be highly variable (Abdel-Farid *et al*., 2007). Moreover, not only the developmental stages but also each organ, part or even cells of the same organ have their own metabolic profile and any oversight of this factor can lead to non-homogeneous sampling and consequently erratic outcomes.

To ensure the reproducibility of experiments it is important to detain any kind of chemical or enzymatic reaction that may occur immediately after sample collection, because these can alter the original metabolite profile of the organism (Koning and Dam, 1992); this rapid inactivation of all biochemical and enzymatic activity in organisms is known as quenching. Cellular metabolism is a dynamic process and any change can alter the metabolic turnover, thus proper quenching can reduce the impact of this problem. The quenching method depends on the goal of what will be analysed or how: for example, whether both the intracellular or extracellular metabolites have to be measured together (microorganisms, cell cultures) or whether the biomass has to be separated from the media. In the first case, a quenching method, which also partially disrupts the cells, can be used as there is no need for media separation, but in the second case, care has to be taken that the quenching method does not destroy the cells, otherwise leakage will affect the original metabolite concentration in the media and within the cells. For this, there are some methods available, including the use of methanol (hot > 80°C or cold < -40°C), which causes a sudden temperature shock and helps to interrupt biochemical reactions. Other methods include changes in pH using perchloric acid, or sudden temperature drops achieved by dipping the sample in liquid nitrogen (Hajjaj *et al*., 1998; Abdel-Farid *et al*., 2007; Teng *et al*., 2009). The choice of the appropriate quenching method also depends on the biological sample to be analysed, as mentioned earlier, because different types of biological samples have different matrixes. In the case of fungi, plants and animals, the tissues are distributed in several layers and peripheral layers tend to be quenched before the inner layers. Thus, care should be taken regarding the sample size and time required to completely interrupt the physiological reactions. Some of the general quenching methods are listed in Table 1.

Table 1. Different techniques for quenching according to the type of metabolite

Organism	Quenching method	Leakage	Separation of media	References
Microbes, cultured cells	Liquid nitrogen	No	No	Tiziani *et al*., 2009
	Cold methanol	Yes	Yes	Koek *et al*., 2006
	Cold ethanol	Yes	Yes	Ewald *et al*., 2009
	Perchloric acid	Yes	Yes	Koek *et al*., 2006
Yeast	Cold methanol	Yes	Yes	Koning and Dam, 1992
	Methanol base buffer (methanol + 10 mM tricine buffer)	Yes	Yes	Castrillo *et al*., 2003
	Liquid nitrogen	No	No	Mashego *et al*., 2003
	Pre-cooled stainless steel beads	No	Yes	Theobald *et al*., 1997
Fungi	Chilled water	Yes	Yes	Matsuzaki *et al*., 2008
	Liquid nitrogen	No	No	Hajjaj *et al*., 1998
	Cold methanol	Yes	Yes	Mashego *et al*., 2007
Insects	Cold methanol	Yes	NA	Bratty *et al*., 2011
	Liquid nitrogen	No	NA	Williams *et al*., 2010
	Cold acetonitrile	Yes	NA	Pedersen *et al*., 2008
Plants	Liquid nitrogen	No	No	Kim *et al*., 2009
Animals	Liquid nitrogen	No	NA	Wang *et al*., 2011
	Cold methanol	Yes	NA	Stentiford *et al*., 2005

NA, not applicable.

Drying and sample storage. Drying is another crucial step in sample preparation before extraction. There are different reasons for this: primarily it facilitates the long-term storage of samples by inhibiting enzymatic activity and microbial growth and furthermore, the presence of water in samples can affect the solvation power of the extraction solvents. Yet another reason for removing water is that it can interfere with the instruments used to analyse the samples, especially in the case of nuclear magnetic resonance (NMR) spectroscopy where traces of water in the samples can distort the resolution of the spectra. Water in samples can also affect gas chromatography (GC) columns, resulting in the loss of reproducibility (Rangarajan and Ghosh, 2011). Samples can be dried by heating, using ovens, microwaves or hot air (Harbourne *et al*., 2009) or by freeze-drying. Among the methods mentioned above, freeze-drying is the best for different kind of samples. When freeze-drying, samples must be frozen with liquid nitrogen first and then transferred to a chamber that is at low pressure and low temperature so that the water in the sample will sublimate. Although it is almost impossible to eliminate the chemically bound water, it does not pose a threat for long-term storage, as it does not participate in microbial growth and chemical reactions (Kim *et al*., 2009). This method is very useful for samples that are sensitive to high temperature. Once the samples are completely dry they should be stored in a controlled environment at −80°C or under liquid nitrogen for example, but in all cases, care should be taken to prevent the reabsorption of water from the surroundings. The choice of drying method depends upon different factors, including the kind and stability of the metabolites to be analysed, the speed at which the sample should be dried and its size. Naturally, the risk of losing volatile compounds should be taken into account.

Homogenisation. Samples should be well homogenised to achieve an effective extraction. This step is very important for metabolomics. As the complexity of an organism increases so does its heterogeneity, as in the case of plants (Kim et *al*., 2009) and animals. Thus, the goal of this step is to achieve sample homogeneity and this can be done by means of either matrix disruption or particle size reduction. Metabolites are present in different compartments of cells, thus the disruption of those cells or their protective covering can maximise the extraction of metabolites. The strength of a cell membrane or cell wall depends upon the interaction of lipids, proteins, carbohydrates, and the degree of polymerisation or bonding between these different molecules. This sort of layering also controls the movement of metabolites across the cells. Thus, a good disruptive method increases the chances of extracting the maximum amount of metabolites from the cells. Different kinds of matrix disruption techniques have been reviewed extensively by Burden (2007).

During matrix disruption it is important to keep the effect of particle size of samples in mind, as it greatly affects the extraction process. Theoretically, the rate of extraction is inversely proportional to particle size. The reason for the increase in extraction efficiency with smaller particle sizes is the dependence of diffusion on the mass or size of the particles. A smaller particle size reduces the time required for the diffusion of solvents, thus facilitating the direct extraction of the metabolites in the solvents (Pfenning *et al*., 2011). Coats and Wingard (1950) reported how the size and shape of particles significantly affected the extraction of oils from seeds and Sari and Velioglu (2011), described how the theanine content determined in tea varied according to the size of the ground tea-leaf particles.

Several techniques that can be used separately or in combination to achieve the purpose of homogeneity and matrix disruption for fast and reliable extraction are available. Among them, the oldest and most conventional method is grinding. It can be done manually with a mortar and pestle, and this is still the gold standard for metabolomics (Lin *et al*., 2007), but this method is laborious and time-consuming. Another alternative is to use homogenisers, in which the sample can be homogenised with liquid if necessary, although this is not the method of choice for small samples (Lin *et al*., 2007). The efficiency of hand-disperser grinding and ball-mill grinding of *Plantago lanceolata* was compared and results showed that the hand-disperser grinding produced a better homogenisation of the sample (Lin *et al*., 2007; Maier *et al*., 2010). The choice of the grinding method depends upon physical characteristics of the material to be ground, for example, large bulbs or vegetables cannot be ground by hand, so laboratory-grade blenders that produce homogeneous samples can be used (Lubbe *et al*., 2011). Care should be taken that whatever method is used, the temperature of the samples remains low enough to avoid the activation of enzymes that could alter the metabolic profile. A widely used method in metabolomics studies is the combination of grinding and ultrasonication, which can be very effective in the extraction of a diverse range of metabolites (Choi *et al*., 2004; Saric *et al*., 2007). Ultrasonication uses acoustic energy to produce low- and high-pressure waves in liquids, leading to the formation and collapse of small bubbles due to strong hydrodynamic sheer forces. Ultrasonication can also be considered as an alternative to high-speed mixers or strongly agitated ball-mills.

Another technique used, especially for small particle size samples such as yeast, cell cultures, microorganisms or body fluids, is freeze–thaw cycles (Zivkovic *et al*., 2009; Smart *et al*., 2010). These cycles can cause the breakage of cell structures allowing metabolites to seep out. Usually the range of cycles is from 1 to 3. However, Teahan *et al*. (2006) suggested that excessive freeze–thaw of samples could affect overall results and produce an artificial separation between the groups so that they should be reduced to a minimum. To achieve a good homogenisation, the above-mentioned techniques can be used separately or combined. Burden (2007) suggested that multistep homogenisation methods are more effective than single-step methods.

Extraction

Once the sample has been collected, dried and stored as discussed previously, it must be extracted. The main goal of extraction is to obtain the maximum number of metabolites or ideally, all the metabolites present in the sample. To achieve this goal, different kinds of extraction methods are used, usually applying different solvent combinations. Here we will discuss the role and effect of different solvents and techniques on the extraction process.

Solvent

The choice of the solvent is a very important and critical step in an extraction procedure. Due to the presence of a wide array of metabolites it is not possible to extract them with one single solvent (Kim *et al*., 2009). There are a number of points that

should be borne in mind when selecting a solvent: toxicity, solubilisation power, selectivity, dissolution rate, chemical reactivity and pH (Schripsema, 2009). Ideally the solvent should be non-toxic and have a high solubilising power. The toxicity of a solvent can be handled by applying routine safety procedures (Maltese *et al.*, 2009), but the solubilising power is an inherent characteristic of each solvent (Table 2). Apart from the solubilisation power – defined as the power or ability of solvent to dissolve a solute – another important feature is solvent selectivity. Solvent selectivity can be defined as the ability of the solvent to dissolve specific compounds, when their polarities are not very different. Snyder (1974, 1978) did a great amount of very valuable work on solvents and their selectivity, particularly for liquid chromatography applications. He calculated a polarity index for different solvents and their molecular interactions and built a very illustrative solvent-selectivity triangle (Table 3). This table shows, for example, that solvents such as diethyl ether and ethoxybenzene have the same P' value but very different interactions with solutes, so they are placed in different selectivity groups. On the other hand, both water and chloroform, having very diverse dipole moments, have a high x_d value (H-donor property) and are thus in a similar group. Snyder suggests the use of the following equation to adjust the polarity of different mixtures to required values:

$$P' = \Phi_a P_a + \Phi_b P_b,$$

in which Φ are volume fractions of solvents in mixtures, and P_a and P_b are the polarity indexes of solvents *a* and *b*.

One way to select the best mixture for a specific task is to test blends of polar and non-polar solvents and calculate their P' values. Once the optimum value has been selected, according to the polarity range of the targeted metabolites, thus providing maximum solubility, the selectivity can be explored by using solvents with the same P' values but different selectivity if necessary.

Thus, the inherent selectivity of solvents must be considered when choosing the extraction solvent and in the case of metabolomics, a non-selective solvent may be the best choice to obtain the largest possible number of metabolites. Compatibility issues with the analytical platform chosen for sample analysis also condition solvent choice as well as the characteristics not only of the metabolites to be extracted but also of the sample matrix.

Table 2. Table of commonly used solvents and their solvent strength values (from Snyder, 1978)

Solvent	Solvent strength
Water	>>1
Methanol	0.95
Ethanol	0.88
2-Propanol	0.82
Dimethyl sulphoxide	0.75
Pyridine	0.71
Acetonitrile	0.65
Ethyl acetate	0.58
Tetrahydrofuran	0.57
Acetone	0.56
Dichloromethane	0.42
Chloroform	0.4
Diethyl ether	0.38
Benzene	0.32
Toulene	0.29
Hexane	0.01

Table 3. Solvents and their selectivity values (adapted from Snyder, 1978)

Solvents	P'	x_e	x_d	x_n
Toulene (VIb)	2.3	0.32	0.24	0.44
Diethyl ether (I)	2.9	0.55	0.11	0.34
Ethoxybenzene (VIb)	2.9	0.27	0.29	0.44
Methylene chloride (V)	3.4	0.34	0.17	0.49
n-Propanol (II)	3.9	0.53	0.21	0.26
n-Butanol (II)	3.9	0.53	0.21	0.26
Chloroform (VIII)	4.4	0.28	0.39	0.33
Acetonitrile (VIa)	6.2	0.3	0.26	0.41
Methanol (II)	6.6	0.51	0.19	0.3
Water (VIII)	9	0.4	0.34	0.26

P' is the polarity index of the solvent and x_e, x_d, *and* x_n represent the fraction of P' contributed by interactions associated with proton-donor, proton-acceptor and dipole moment characteristics of the solvent, respectively.

Solvent-based extraction

Acid-base extraction. Perchloric acid extraction is a widely used extraction method for animal samples (Rammouz *et al.*, 2010) and is also applied for plants, particularly to study primary metabolites (Kruger *et al.*, 2008). It is a strong oxidising acid, and is comparable in acidity to hydrochloric acid. One of the aims of the preparation of samples for metabolomics is the elimination of high molecular mass compounds (macromolecules) such as proteins, because they can have deleterious effects on analytical determinations. Perchloric acid extraction denatures proteins, yielding thus, a protein-free extract. The main steps in this kind of extraction include the: (i) extraction of metabolites with an aqueous perchloric acid solution; (b) neutralisation of the pH of the resulting sample with a potassium carbonate or hydroxide solution; (c) removal of the perchlorate salts formed during the neutralisation step. Naturally, this kind of extraction is appropriate only for metabolites that are stable under acidic conditions. A major disadvantage of this method is the need to adjust the pH for each sample before measuring, sometimes leading to a great variation between samples, especially in case of NMR analyses (Lin *et al.*, 2007). Other disadvantages include the presence of perchlorate salts, which if not completely removed can be incompatible with liquid or gas chromatography coupled to mass detectors (LC–MS or GC–MS). Furthermore, the difficulty to adjust the pH of every sample to exactly the same value can lead to different elution times or peak shifting, complicating data-mining techniques. Thus, the main and perhaps, yet only significant advantage of perchloric acid extraction is the denaturation of proteins and total inactivation of enzymes (Kruger *et al.*, 2008). This kind of extraction technique will allow only the polar, hydrophilic or basic compounds to be studied, as hydrophobic neutral or acidic compounds cannot be extracted. It is particularly useful to study primary metabolites.

In metabolomics. This kind of extraction is used in plant metabolomics. Avelange-Macherel *et al*. (2006) applied this method for a study of cucumber metabolites, using 70%(v/v) perchloric acid to quench the lyophilised samples and then 6% perchloric acid to maximise the extraction of metabolites. The pH of the samples was then adjusted to 5.0 with 2 M K_2CO_3, followed by centrifugation to remove perchlorate precipitates along with proteins. The supernatant was lyophilised before the addition of a buffer adjusted to a pH of 7.5 with concentrated potassium hydroxide or hydrochloric acid. Deuterated water (D_2O) was added at the end for NMR analysis. Brouquisse *et al*. (2001) implemented a slightly modified version of this method, extracting the material with 1.1 M perchloric acid first and then with water. The supernatant was then mixed with 5 mL of 2 M $KHCO_3$. Kruger *et al*. (2008) extracted plant sample metabolites – mostly organic acids, sugars, amino acids and some aromatic compounds – with a 1.0 M perchloric acid solution. Observing the perchloric acid fraction of a plant material, Kaiser *et al*. (2009) concluded that this acid had caused the hydrolysis of sugars, especially of sucrose, thus affecting the biological interpretation of samples, as erroneously high levels of glucose and fructose were observed.

Perchloric acid extraction is an extensively used method in animal metabolomics due to its advantage of immediately quenching enzymatic reactions and because of their high protein content as compared to plants. The protocol generally consists in the extraction with 6% or 0.5 M perchloric acid followed by the addition of a basic solution to neutralise the pH, lyophilisation and then resuspension in the desired solvent (Ekman *et al*., 2006; Rammouz *et al*., 2010). Griffin and Kauppinen (2006) reported that perchloric acid is widely used for extracting brain metabolites due to its strong quenching ability, but its oxidising properties and the presence of high amounts of salts resulting from the acid-base reaction could be problematic for mass spectrometry. Lin *et al*. (2007) compared the extraction of salmon muscle with ice cold 6% perchloric acid, acetonitrile: water (1:1), acetonitrile:water (2:1), methanol:water (1:1), methanol:water (2:1), and the biphasic systems, methanol–chloroform–water, or methanol–chloroform–water with 0.8% KCl. Following extraction and centrifugation, the supernatants of all organic extracts were removed and liophilised, while the perchloric acid extract had to be neutralised with potassium carbonate, left on ice and then centrifuged again before lyophilisation. They reported a significant shift of peaks in the NMR spectra of samples extracted with perchloric acid (in spite of having buffered the samples) of metabolites with a p*Ka* value near 7.2. Although perchloric acid extraction also leads to poor recovery of certain metabolites, as explained previously, it is still a useful method for targeted analysis (Sellick *et al*., 2009; Rammouz *et al*., 2010).

In the case of microorganisms or cell cultures, it is important to use a method that prevents the leakage of intracellular metabolites in order to obtain the most reliable results. In a study conducted by Faijes *et al*. (2007) on targeted metabolites (ATP, ADP and AMP) the use of pre-cooled perchloric acid as an extraction solvent proved to be good in terms of the recovery of these metabolites, together with cold methanol as a quenching solvent causing minimal leakage of ATP from the cells. However, Müller *et al*. (1996) compared the results of pre-cooled 1.3 M perchloric acid extraction with an alkaline extraction method, and concluded that the neutralising step could cause a time-dependent decrease in nucleotides, while perchloric acid extraction also interfered with ion-pair reversed-phase HPLC due to the presence of traces of perchlorate ions. Schaefer *et al*. (1999) and Buchholz *et al*. (2001) successfully used 35% w/v perchloric acid for the extraction of glycolytic intermediates, and tricarboxylic acid pathway metabolites. For yeast cells, Theobald *et al*. (1997) and Buziol *et al*. (2002) used 35% pre-cooled perchloric acid (−25°C) for the determination of intracellular metabolites. Lafaye *et al*. (2005) also reported the use of boiling 0.1% perchloric acid (pH 2–2.5) for the extraction of sulphur-containing metabolites from yeast followed by a HPLC analysis. Hajjaj *et al*. (1998) used 10% v/v pre-cooled perchloric acid for the extraction of the filamentous fungi *Monascus ruber* and reported a low recovery of acid stable metabolites along with a significant loss in nucleotide concentration.

Organic solvent-based extraction

The above-mentioned perchloric acid extraction method is suitable for the extraction of polar, hydrophilic or ionisable metabolites. In the case of moderately polar, non-polar or hydrophobic metabolites, organic solvents must be included. One of the main advantages of using organic solvents rather than water is that more diverse types of metabolites can be extracted. In the case, for example, of a two-phase solvent system with water–methanol–chloroform, not only polar, hydrophilic metabolites but also non-polar compounds could be extracted simultaneously. Other advantages include the compatibility of most organic solvents with all analytical platforms including GC–MS, HPLC, LC–MS, NMR and Capillary electrophoresis (CE), the ease of solvent evaporation, absence of salt precipitates and increased stability of the extracted metabolites.

Among the many options, alcohols, especially methanol, ethanol and in lesser degree isopropanol are often used, alone or more usually in mixtures with varying proportions of water. Being hydrophilic due to their hydrogen-bonding capabilities, alcohols are also lipophilic, a property that increases with their chain length; the combination of these features confers a high solubilisation power that allows them to extract a wide range of metabolites.

In metabolomics. Mixtures of methanol and water are the most popular combinations for metabolomic studies because they have proven to be able to extract a wide range of metabolites, namely, sugars, amino acids, organic acids, alkaloids and phenolic compounds. Their 'non-selectivity', as discussed above, makes them especially suited to metabolomics applications and most plant metabolites can be extracted in these solvents. Isopropanol has been used for LC–MS metabolite profiling studies targeted to more lipophilic compounds, that is, to evaluate jasmonate synthesis or the role of jasmonic acid signalling as a response of *Arabidopsis thaliana* leaf to wounding (Grata *et al*., 2008; Chauvin *et al*., 2013). The use of this solvent increases the extraction of more lipophilic metabolites but also requires the use of solid-phase extraction (SPE) removal of unwanted non-polar compounds such as chlorophylls and other substances that would reduce the life of reversed-phase columns.

De Vos *et al*. (2007) used acidified water (0.1% formic acid) in 75% methanol (pre-cooled) to extract a wide range of secondary metabolites (flavonoids, phenolic acids, alkaloids and glucosinolates) and reported it to be a very efficient method. This did not coincide with the conclusions of Bertrand *et al*. (2013), who found that the extraction efficiency of a number of solvent

systems decreased dramatically with the addition of 1% formic acid. Anthocyanins also were extracted with 75% methanol from tomatoes and analysed on a LC–MS-based platform by Moco *et al.* (2006). An NMR-based analysis of *Brassica rapa* metabolites was conducted by Jahangir *et al.* (2008) using a direct extraction in deuterated methanol and water to analyse the changes in glucosinolates, hydroxycinnamic acids, carbohydrates and amino acids. Scarcely polar, hydrophobic compounds such as terpenoids, fatty acids or lipids have been extracted with the lipophilic solvents, chloroform, ethyl acetate or *n*-hexane (Cordeiro *et al.*, 1999; Yu *et al.*, 2011). The use of biphasic solvent systems is also quite frequent because they allow the separation of metabolites that could interfere in the analysis of samples, facilitating their identification. For example, the extraction of metabolites of *Catharanthus roseus* leaves using a very usual combination of chloroform–methanol–water for ^{1}H-NMR analysis, allowed indole alkaloids to be clearly detected in the chloroform phase, separated from primary and secondary metabolites such as loganic acid and secologanin that remained in the aqueous–methanolic phase. The characteristic signals of vindoline in the δ 5.8–7.0 region would overlap with these signals if all compounds were together in a mid-polar solvent extract (Choi *et al.*, 2004). However, some practical issues deriving from the presence of saponins, for example in plant samples, which cause emulsions in the interphase and other consequences, such as the need to concentrate and redissolve the extracts, often outweigh the benefits of this type of extraction in plants. In the case of ^{1}H-NMR plant metabolomics studies, the direct extraction of samples, using methanol-d_4-KH_2PO_4 has proved to be efficient and is a widely used protocol (Liang *et al.*, 2006; Simoh *et al.*, 2009). Naturally, this solvent system cannot be used for LC–MS and when comparing the metabolite diversity of induced co-cultured fungi extracts of methanol, isopropanol, t-butanol, dichloromethane:methanol:water (64:36:8) and dichloromethane:ethyl acetate:methanol (2:3:1) with or without 1% (v/v) formic acid, Bertrand *et al.* (2013) found that the best results were obtained with the biphasic system. Some examples of different solvent systems along with the type of metabolite extracted are listed in Table 4.

As animals have a higher lipid and protein content and less secondary metabolites than plants, the protocols used for animal metabolomics extraction are not as diverse as those for plants. The high lipid content is dealt with using biphasic systems similar to those mentioned above, that is, methanol, chloroform and water. Again, the ratio depends upon the type of metabolites to be extracted. The removal of proteins can be done using perchloric acid extraction as discussed above, but when relatively non-polar metabolites are targeted, or in the case of fatty tissues, organic solvents can be more efficient. One study (Rammouz *et al.*, 2010) compared different extraction strategies using LC–MS as the analytical platform and suggested that boiling water is the best way to analyse the skeletal tissues except creatine, which showed a poor recovery. Considering the recovery of the metabolites, he graded the methods as follows: boiling water (100°C) > methanol–water–chloroform > methanol–water > cold pure methanol (−80°C) > perchloric acid (−20°C) > boiling ethanol (80°C). Lin *et al.* (2007) compared several solvent systems for the extraction of fish liver, consisting in different proportions of acetonitrile or methanol and water and a biphasic sytem of methanol–chloroform–water, recommending the use of the latter, especially in the case of lipid-rich tissues such as liver and brain. They concluded that although single-solvent extraction methods have the advantage of their ease and reproducibility, if mixtures of hydrophilic and hydrophobic metabolites have to be recovered, the best results would be obtained with the biphasic system, despite some disadvantages such as the time and care

Table 4. Organic solvents used for the extraction of different types of compounds from plant material

Plant	Solvent method	Compounds extracted	Analytical platform	Reference
Solanum lycopersicum	75% Methanol	Anthocyanins	LC–MS	Moco *et al.*, 2006
Solanum tuberosum	62.5% Methanol 0.1% Formic acid Methanol, water, chloroform system	Hydroxycinnamic acids Catecholamines	LC–MS GC–MS	Szopa *et al.*, 2001; Vorst *et al.*, 2005
Brassica oleraceae	60% Methanol Hot 70% Methanol Cold methanol	Glucosinolates, Phenolic acids, Flavonoids Sinapic acid derivatives	HPLC CE, HPLC UPLC/DAD–MS/MS	Robbins *et al.*, 2005; Gratacos-Cubarsi *et al.*, 2009; Lee *et al.*, 2010
Brassica rapa	CD_3OD: D_2O(1:1)	Glucosinolates, amino acids, sugars	NMR	Jahangir *et al.*, 2008
Maytenus aquifolium *Maytenus ilicifolia*	Ethyl acetate at 40°C	Terpenoids	HRGC–MS	Cordeiro *et al.*, 1999
Origanum dictaminus *Teucrium polium* *Lavandeula vera*	62.5% Methanol Ethyl acetate	Phenolic compounds	HPLC, GC–MS	Proestos *et al.*, 2006
Cataranthus roseus	Two-phase solvent system (MeOD, D_2O)	Phenolic conpunds Organic acids	NMR	Choi *et al.*, 2004

required to transfer the phases, which limits its use in high throughput sample processing. Table 5 shows different solvent systems and the type of compounds extracted from mammalian tissue.

As discussed above, both microorganisms and cells are cultured in external media and require rapid quenching. They produce two types of intracellular and extracellular metabolites. Methods to extract metabolites from both kinds of matrices will be discussed here briefly.

Intracellular metabolites. Microorganisms are quite diverse in their nature and produce a wide range of metabolites. In microbes, the ability to produce secondary metabolites is spread unevenly, mainly due to their dependence on their environment rather than phylogeny (Vining, 2007). Because of this diversity, different kinds of systems have been applied to extract the maximum number of metabolites, but as yet no single method has proved to be globally efficient for all metabolites. However, advances in technology have allowed researchers to identify 100–500 metabolites in one single analysis of a microorganism (van der Werf *et al*., 2007).

The most commonly used solvents for intracellular metabolite extraction are ethanol, methanol and chloroform (Table 6).

Extracellular metabolites. Extracellular metabolites are those that are secreted by the organism during its life cycle. Thus, their analysis can provide a good insight into how the organism interacts and responds to its environment. One of the easiest methods to separate cells from the medium is centrifugation (Sulek *et al*., 2011). Another method is filtration, but this can be quite difficult due to the presence of viscous material in growth media (Devantier *et al*., 2005). After removal of the cells, the supernatant can be either quenched or lyophilised, and extracted with any suitable solvent system. The medium can be separated from the metabolites with a non-water miscible solution or by using solid-phase extraction.

Different techniques in extraction

Microwave-assisted extraction

The first use of microwave-assisted extraction (MAE) for organic compounds was reported by Ganzler *et al*. (1986), who successfully applied it to partition and extract various types of compounds from a range of different samples (baby food, animal fodder, maize). In MAE, the solute is in direct contact with solvents heated by microwave energy (2.45 GHz), and this kinetics-driven elevated temperature causes the rapid extraction of desired compounds with a minimum amount of solvent. A typical extraction procedure takes 15–30 min, with solvent volumes that range from 10–30 mL (Sparr Eskilsson and Bjorklund, 2000). Two versions of MAE are available: focused open-vessel MAE (FOV–MAE) or closed-vessel MAE (CV–MAE). The main difference between the techniques is the instrumentation. In FOV–MAE, the vessel containing the sample and solvent is open whereas in CV–MAE it is closed. Both have advantages and limitations. The main advantages of using CV–MAE are that pressure is controlled and an increase in temperature is achieved in less time with no loss of volatiles or any need to add extra solvent. The limitations are that sample size is limited and the risks derived from high pressurisation. On the other hand, the main advantage of FOV–MAE is the possibility of using large amounts of sample and of adding reagents at any time. The limitations for this method are a lower precision and longer extraction times than those required by CV–MAE. Before using MAE there are few factors that should be taken into account.

Solvent system. As in any kind of extraction method, the solvent plays a major role in MAE and will affect the efficiency of the method. The choice of the solvent depends upon the interaction of the solvent with the matrix, the solubility of the desired analyte and the microwave absorption ability. The solvent selected for MAE should: be compatible with the matrix of the sample; able to solubilise the target analyte/s; and have microwave absorption capability. The microwave absorption ability of solvents depends upon their dielectric constant: in theory the larger their dielectric constant, the larger their ability to absorb energy and become hot (Dean, 2009). A few solvents with their corresponding dielectric constants are shown in Table 7.

As can be observed, water has the highest microwave absorption whereas hexane is literally transparent to microwaves and thus does not heat up (Tatke and Jaiswal, 2011). Solvents can be used separately or in combinations, depending on the nature of the experiments. Solvent properties can be modified using mixtures of different polarities and dielectric constants to make them more suitable for different compounds. For example, in

Table 5. Application of different organic solvent systems for animal tissue sample extraction considering the platform used for the metabolomic study

Matrix	Solvent system	Compound extracted	Platform	References
Rat brain	1% Formic acid Cold water Methanol–water mixture	Amino acids Organic acids Neurotransmitters Catecholamines	UPLC–MS/MS GC–MS	Kanani *et al*., 2008; Goh *et al*., 2009
Mice liver	Methanol–chloroform (2:1)	Glycerols, esters, phospholipids	HPTLC LC–MS	Van Ginneken *et al*., 2007
Oesophagous cancer tissue	Methanol–water–chloroform (2:5:2)	Organic acids, amino acids, sugar derivatives, free fatty acids, nucleosides	GC–MS	Wu *et al*., 2009
Mice liver Nematodes	Methanol/chloroform (2:1)	Amino acids Free fatty acids	NMR GC–MS	Atherton *et al*., 2008
Fish	Chloroform/methanol (3:1)	Lipids, free fatty acids	NMR	Samuelsson *et al*., 2006
Ground squirrels	Methanol	Acyl esters, amino acids	LC–MS	Nelson *et al*., 2010

Table 6. Application of organic solvent extraction for metabolomic studies of microorganisms and cell cultures

Species	Solvent	Metabolites	Platform	References
Escherichia coli	Boiling ethanol Methanol, chloroform, water (1,1,0.5 respectively) at −45°C	Amino acids, nucleotides	TLC GC–MS GC–FID	Tweeddale *et al*., 1998; Tian *et al*., 2008
Mycobacterium tuberculosis	Acetonitrile:methanol:water (40:40:20) at −40°C	Glycolysis intermediates, TCA intermediates	LC–MS	De Carvalho *et al*., 2010
Xanthomonas campestris	80% Methanol	Glycolysis intermediate,	GC–MS	Neuweger *et al*., 2008
Saccharomyces cerevisiae	Chloroform Methanol at −80°C	Glycolysis intermediate	Enzymatic GC–MS	Koning and Dam, 1992; Devantier *et al*., 2005
SW cells colon cancer cells	75% Methanol at −20°C	Osmolytes, amino acids, nucleotides	NMR	Maddula and Baumbach, 2010
Fusarium oxysporum	Methanol, chloroform at −20°C	TCA cycle intermediates, Amino acids and dibasic acids	CLC	Panagiotou *et al*., 2005
Coprinus disseminates *Coprinellus micaceus* *Stereum hirsutum*	Methanol at 4°C	Hydroxy quinones, organic aicds, free fatty acids, sugars	TLC GC–TOF MS	Peiris *et al*., 2008

Table 7. Common solvents used in microwave-assisted extraction: boiling and melting points and dielectric constants (at 20°C)

Solvent	Melting point (°C)	Boiling point (°C)	Dielectric constant (K)
Acetone	−95	56	20.7
Acetonitrile	−44	82	37.5
Chloroform	−64	61	4.81
Hexane	−95	69	1.89
Ethanol	−114	78	24.5
Methanol	−98	65	32.7
Water	0	100	80.1

the case of volatile compounds, solvents with a low dielectric constant can be used to ensure they are exposed to low temperatures and will cool faster once they are extracted (Routray and Orsat, 2011).

Volume of solvent. Generally, the volume of solvents used for MAE is in the range of 10–30 mL. This volume should be sufficient to cover the sample completely, taking into account any swelling during extraction (Sparr Eskilsson and Bjorklund, 2000). For example, Zhu *et al*. (2006) used 20 mL of solvent for 400 mg of a tobacco sample for the extraction of volatile compounds. Misra *et al*. (2013) reported that an increase of solvent from 5 to 10 mL slightly increased the recovery of artemisinin from dried leaves of *Artemisia annua*. Generally, increasing the volume of the extraction solvent usually leads to a higher recovery of the solutes, but in the case of MAE this might not be always true. Li *et al*. (2004) found that during the extraction of geniposidic and chlorogenic acid from *Eucommia ulmodies* an increase in solvent volume actually reduced the recovery of the desired compounds. Thus, in the optimisation process of MAE, the volume of solvent should be borne in mind as an important factor.

Temperature. Temperature is another very important and well-studied factor in MAE, because as for most extraction techniques it significantly affects recovery. Especially when MAE is conducted in a closed vessel, the temperature of the solvents may be heated to ca. 100°C above their atmospheric pressure boiling point. This high temperature results in a decreased viscosity and surface tension and increases the adsorption of the solvent to active sites of the solute (Kaufmann and Christen, 2002). However, the effect of temperature is not always predictable: Jin *et al*. (2010) showed that increasing the temperature up to 30°C increased the yield of tannins from the Chinese herb *Argimonia pilosa* but a further increase decreased the yield. A similar effect was observed for the recovery of polyphenols from the fruit hull of *Camellia oleifera* (Zhang *et al*., 2011).

Microwave power. Microwave power provides localised heating to the matrix and can actually even destroy it, thus assisting the diffusion of the analyte into the solvent (Chan *et al*., 2011). Generally, the choice of microwave power, especially in the closed vessel system, depends upon the number of vessels or the total amount of liquid used for extraction. Ideally, the power used should minimise the time required to reach the set temperature without producing the bump phenomenon (Sparr Eskilsson and Bjorklund, 2000). Low or moderate temperatures with longer times of exposure can be selected to optimise MAE (Tatke and Jaiswal, 2011). Young (1995) reported that the use of 10% of the maximum power (750 W) for 3 s resulted in a considerable yield of ergosterol and an increase of up to 50% of the power provided the highest yield, but a longer exposure to the maximum power (750 W) caused tube leakage. It

has been observed that once microwaves have destroyed the matrix, a further increase in power does not improve the yield significantly (Alfaro *et al*., 2003). Shu *et al*. (2003) also reported that the use of different levels of microwave power is more significant when applied for shorter periods than for longer extraction times. The danger of using high power for long extraction times is, among other things, the thermal destabilisation of thermolabile compounds (Xiao *et al*., 2008).

Extraction time. Prolonged extraction times, even at moderate power, can result in decreased yields and the degradation of active compounds (Hao *et al*., 2002; Wang *et al*., 2009). Extraction time in MAE is quite variable and ranges from a few minutes (Pastor *et al*., 1997) to hours, especially in the case of solvent-free MAE in which more time is required to extract analytes (Lucchesi *et al*., 2004). The risk of degradation due to long extraction times can be overcome by introducing shorter extraction cycles, that is, adding fresh solvent to the residue and repeating the extraction. Xiao *et al*. (2008) showed that two extraction cycles are sufficient to extract flavonoids completely from *Radix astragali*. Similarly, Shao *et al*. (2012) observed that two extraction cycles yielded 96% flavonoids from *Perilla frutescens* leaves, whereas a third cycle yielded only an additional 4%.

In metabolomics. Microwave extraction is more popular for plant than for animal metabolomics studies, probably because the diversity of metabolites is much greater in plants and their extraction is more challenging. Compared with the use of conventional methods to extract different classes of compounds such as flavonoids and phenols, which are time consuming and difficult, the use of MAE certainly reduces the time. Proestos and Komaitis (2008) compared the conventional reflux method for phenolic compound extraction with MAE, using different solvent systems, and reported that not only were extraction times reduced but also the extraction efficiency of different solvents was increased.

As the awareness of the damage caused by toxic solvent residues increases, researchers in the field of metabolomics are considering the option of using green solvents. The use of such solvents (ionic liquids and water) has shown to be efficient and safe as compared with other conventional solvents for the extraction of secondary metabolites from plants. Du *et al*. (2009) successfully applied MAE with ionic liquids (ILs) for the extraction of phenolic compounds from *Psidium guajava* leaves and *Smilax china* tubers, reporting that the use of ILs together with MAE could efficiently replace the conventional reflux method with methanol, as ILs with MAE not only reduced the extraction time, but also the waste.

Although MAE is mostly used in plants, there are a few studies involving clinical samples such as blood, hair and urine where it was used. Keller *et al*. (2009) studied blood samples for organohalogen contaminants by using different extraction techniques such as liquid–liquid extraction (LLE), SPE, pressurised fluid extraction (PFE) and focus microwave assisted extraction (FMAE), finding that among the different methods, FMAE showed the greatest recovery. Fernández *et al*. (2009) successfully extracted cocaine and its metabolites from human hair samples using MAE. To date, no study has been reported for global profiling of animal and human metabolomics using MAE, the reason probably being that there are always thermolabile metabolites that could be destroyed using this method (Teo *et al*., 2013).

Supercritical fluid extraction

Supercritical fluid extraction (SFE) can be defined as the separation of one component from others by the use of a supercritical fluid. A supercritical fluid is a liquid that is above its critical temperature and pressure. Charles Cagniard first discovered supercritical fluids in 1822. They have, or display, properties of both gases and liquids, diffusing into a solid like a gas while dissolving substances like liquids. Small changes in temperature and pressure can dramatically change the properties of supercritical fluids, thus allowing control of their physicochemical properties (density, solvation power) (Abbas *et al*., 2008). There are two main applications of SFE technology: (i) countercurrent extraction from liquids streams; and (ii) extraction from solid particles (Oliveira *et al*., 2011). Countercurrent SFE has been applied to modify essential oils, for example, the deterpenation of orange peel oils (Espinosa *et al*., 2005) or obtaining high-value products from waste such as the purification of squalene from vegetable oils (Vázquez *et al*., 2007). On the other hand, the decaffeination of coffee beans with SFE is an example of the extraction through solid particles or a bed. After the extraction process is finished the solvent is easily removed from the extracted material by simple expansion. This, together with the ease of manipulating solvation characteristics by slight changes in gas and temperature of the supercritical fluid are among the main advantages of SFE over conventional methods.

A few of the substances that can be used as SFE solvents are listed in Table 8. Among them, carbon dioxide is clearly the most popular. Some of the reasons for its choice are its low critical temperature and pressure, high purity, low cost, easy recovery and low toxicity. Additionally it is almost inert in the standard operating conditions, that is, 304 K and 7.37 MPa. It is also convenient for the extraction of heated labile and non-polar compounds. The main disadvantage of CO_2 is its non-polar nature, but this can be overcome by adding modifiers or co-solvents, as will be discussed later. Carbon dioxide SFE (CO_2-SFE) has been shown to have a wide range of applications, not only for extractions but also in purification processes. In the field of natural products, CO_2-SFE has long been used to extract bioactive compounds. Phenols and sesquiterpenes have been extracted either using pure CO_2 or CO_2 with the addition of some methanol as a modifier (Zizovic *et al*., 2007; Sun *et al*., 2008). Díaz-Reinoso *et al*. (2006) extensively reviewed the extraction and purification of anti-oxidants from natural sources using CO_2-SFE. Some other substances have also been used as SFE solvents. For example, Nobre *et al*. (2011) used and compared ethane with CO_2-SFE for the extraction of lycopene from industrial tomato

Table 8. Critical properties of solvents used for superfluid critical extraction

Solvents	CT (K)	CP (MPa)	CV (cm^3/mol)
Ammonia	405.4	11.35	72.5
CO_2	304.1	7.37	94.1
Methanol	512.6	8.09	118.0
n-Hexane	507.5	3.02	368
Water	647.1	22.06	55.9

CT, critical temperature; CP, critical pressure; CV, critical volume.

waste and found it to be more efficient in terms of extraction speed and recovery. Because ethane is more expensive than CO_2, the use of mixtures with the cheaper CO_2 has also been proposed. Propane and dimethyl ether have been tested for the extraction of pungent compounds from ginger, black chilli and pepper, and propane was found to be the least effective of the two (Catchpole *et al*., 2003).

Besides the type of solvent used in SFE, the ability to remove the solute from the matrix depends upon the adjustment and optimisation of the temperature and pressure, solvent flow rate and the influence of co-extractors or modifiers.

Temperature and pressure. As mentioned earlier, the manipulation of both temperature and pressure can significantly affect the physicochemical properties of the solvents, which in turn affect their extraction or solvating power. Phelps *et al*. (1996) suggested that changing both the temperature and pressure of fluids could modify the solvating power of organic solvents. As a rule, an increase in temperature at constant pressure reduces the solvent density and its solvating power, whereas an increase of pressure at constant temperature enhances the solvation power (Pereira and Meireles, 2010). This means that the solubility of a solute in a matrix depends directly on the density of the solvents used. Thus, the solubility of a solute can be manipulated by changing the temperature or pressure conditions.

Solvent flow rate. Solvent flow rate is also an important factor for SFE. Generally, an increase in flow rate increases the extraction capacity but in some cases very high flow rates can decrease it. Kumoro and Hasan (2007) studied the effect of solvent flow rate on the extraction of andrographolide from *Andrographis paniculata* and found that very high flow rates resulted in a lower extraction capacity. The reason for this could be that the contact time between the solvent and the sample is insufficient, considering the low diffusion rate of the solvent/solute within the matrix. In another study, Nemoto *et al*. (1997) analysed the effect of the CO_2 flow rate on the extraction of pesticides and proposed that low flow rates result in low linear velocity and increase extraction efficiency.

Modifiers or co-extractors. As mentioned earlier, CO_2-SFE has one major drawback: the non-polarity of carbon dioxide. A common practice to overcome this problem is the addition of modifiers or co-extractors. A modifier can be added with the solvent or together with the sample in the extraction chamber. The first method can be applied to both countercurrent SFE and static SFE, whereas the second one is always used for static SFE. These modifiers can range from ethanol, acetonitrile and methanol to water. Several mechanisms that may explain modifier activity have been proposed. One theory is the existence of a solute–modifier interaction, in which the modifier interacts chemically with the solute (Pereira and Meireles, 2010) and another is that the modifier might form a solvation shell around the solute (Yonker *et al*., 1986). Generally, the concentration of the modifier ranges between 1% and 15%. Among the different modifiers, methanol and acetone are the most popular (Phelps *et al*., 1996). Choi *et al*. (2000) compared the extraction efficiency of different modifiers for the CO_2-SFE of cephlotaxine from *Cephlotaxus wilsoniana* and concluded that methanol alkalinised with dimethylamine increased the extraction of cephlotaxine by supressing the ionisation of the hydrophilic salt, thus increasing the affinity of the resulting lipophilic base with CO_2. Another example is the solubility of beta-carotene, a compound that should be easily soluble in this solvent, considering it non-polar nature. However, its large molecular weight (536.87 g/mol) inhibits its solubility in CO_2-SFE due to its low volatility (Sun and Temelli, 2006). This problem can be solved by the addition of co-solvents, as shown by the increased solubility achieved by the addition of 5–10% of ethanol (Barth *et al*., 1995). Marsili and Callahan (1993) also used CO_2-SFE with ethanol as a modifier to extract and compare the extraction efficiency of beta-carotene with LLE, concluding that CO_2-SFE not only increased the yield by 23% but was also less time consuming. Vegetable oils were also used as modifiers to increase the extraction of carotenoids and lycopenes in CO_2-SFE (Sun and Temelli, 2006; John *et al*., 2009). The type of modifier to be used should be selected according to its affinity with the solute to be extracted.

In Metabolomics. As mentioned above, SFE is a fast, reliable and highly manipulable extraction technique and its utilisation in metabolomics studies is thus quite widespread. It has the additional advantage of being able to extract compounds with few online adjustments, which would otherwise be difficult to achieve using conventional metabolomics extraction techniques. A few examples of its utilisation in the metabolomics field are mentioned here. Hou *et al*. (2010) used CO_2-SFE to extract alkamides and phenolic compounds from Echinacea plants with the objective of applying comparative metabolomics for quality control and bioactivity validation. This approach was also proposed by Blanch *et al*. (1999) as a method for the detection of highly volatile compounds, which are usually lost during the extraction and fractionation procedures, using a combined CO_2-SFE/GC system. Anticipating that during a conventional SFE extraction procedure some highly volatile compounds could co-evaporate from the collection vessel during the concentration step, he added a programmed temperature vaporiser (PTV) injector between the CO_2-SFE extraction set-up that allowed him to collect and detect the volatile compounds with GC. In another study Uchikata *et al*. (2012) were able to detect 134 phospholipids from a 3-μL dried blood sample spot within 5 min using SFE/supercritical chromatography (SFC) combined with tandem mass spectrometry (MS/MS) and suggested that it could be a useful technique for lipid profiling and biomarker discovery.

Solid-phase extraction (SPE)

Solid-phase extraction is often not considered to be strictly an extraction method, because it is used more often for the clean up and/or concentration of a diverse a range of metabolites from a liquid matrix. In SPE a sorbent is used to adsorb (Fig. 1) the metabolites from a liquid phase (e.g. extracts, mediums or body fluid). Depending upon the affinity and interaction of metabolites with the solid phase, these are then desorbed with a suitable solvent. This technique has been automated and coupled with online extraction and analytical instruments such as GC or LC–MS (Louter *et al*., 1999; López-Blanco *et al*., 2002). Before selecting any kind of cartridge it is necessary to bear in mind the points shown in Table 9, because they can significantly affect the time, cost and recovery of the analyte.

When choosing the sorbent it is very important to take into account the properties of both the matrix and the compound to be extracted. A wide range of sorbents is available on the market and these are well reviewed by Masque *et al*. (1998).

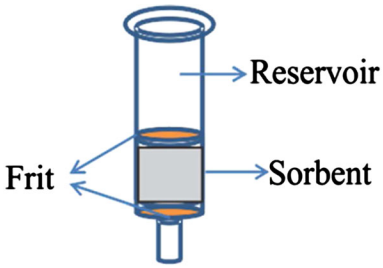

Figure 1. A typical Syrische-barrel type of cartridge, with a frit and sorbent. The sample is added to the barrel and impregnates the sorbent, after which it is flushed with the selected solvent.

Table 9. Type of formats available for solid-phase extraction and factors to be considered before selecting a specific type of matrix (data adapted from Hennion, 1999)

Types	Comments
Syringe barrel/cartridge	Coagulation of sediments at the top Filtration takes a long time Can be corrected by addition of diatomaceous earth
Disc type	Higher flow rate Sediments may cause clogging of channels Glass fibre matrix or in-depth filters can be used to overcome this problem
96-well plate with disc	Initially developed to help high throughput screening and automation
Pipette tips with sorbent	To speed up the automation and reduce the void volume
Microfibre	For head-space analysis by GC–MS

In metabolomics. There are not a great number of applications of SPE as an extraction method in plant metabolomics, because the selectivity of this method is greater than solvent-based extraction methods, so it is generally preferable to use these other methods for extraction and eventually clean up the extract, eliminating interfering compounds with SPE.

Ali *et al*. (2011a,2011b) compared SPE with direct extraction of the phenolic compounds from grapes using deuterated methanol and water, finding that SPE extracts showed increased signal intensities of phenolics in ^{1}H-NMR spectra. They also successfully applied SPE to remove sugars from grape samples to improve spectra resolution. Exarchou *et al*. (2003) used an online SPE system in a LC/UV/SPE–NMR–MS set-up for the analysis of monoterpenes, flavonoids and phenolic acid in Greek oregano, adding protonated solvents in the SPE part for the online preparation of NMR samples.

The vast majority of applications of SPE in metabolomics studies refer to the extraction of metabolites from biological fluids or elimination of proteins before LC–MS-based metabolomic studies. Michopoulos *et al*. (2009) applied SPE to remove proteins from human blood plasma and compared this method with solvent-based precipitation. The UPLC–MS results showed that the reproducibility of the system increased with SPE as compared with other precipitation-based methods.

Solid phase micro-extraction (SPME) has been used extensively in biological fluid metabolomics studies. Dixon *et al*. (2011) applied SPME for the study of faecal volatile organic compounds (VOCs) using GC–MS and GC with Flame ionisation detector (FID) analytical platforms. Similarly, in another study, VOCs from human skin were collected by directly exposing SPME fibres to the skin and then injected into a GC–MS at 230°C for 1 min to desorb VOCs before their analysis (Gallagher *et al*., 2008). Vuckovic *et al*. (2010) wrote a comprehensive review on SPME applications.

Soxhlet extraction

Soxhlet extraction is one of the oldest methods for solid–liquid extraction. Among the other techniques used, it has been the most cited, probably because up to now the optimisation of new extraction techniques often regarded it as the reference. Initially, it was developed to extract lipids from solids (milk) (Soxhlet, 1879). A Soxhlet apparatus consists of a solvent reservoir, an extractor body, an electric heat source and a water-cooled reflux condenser. The sample is placed in a thimble that is then positioned in the extractor body. The solvent is poured into a round bottom flask (solvent reservoir) and the extractor body and the condenser are assembled on top. The solvent reservoir is then placed on a heat source and as the temperature increases the vaporisation process of the solvent starts. Once the solvent reaches the condenser it condenses and pours down into the extractor body, coming into contact with the thimble that contains the sample. As the process continues, the sample is macerated with the solvent until it reaches a certain volume, after which it is siphoned back into the solvent reservoir flask along with extracted solute. At the end of each cycle the concentration of the analyte in the solvent reservoir increases. As the analytes have a higher boiling temperature than the solvent, only pure solvent will reach the sample-containing thimble thereby increasing extraction efficiency. The clever feature of this system is that each cycle consumes small amounts of solvent. This process can continue for a number of cycles (four cycles per hour depending on solvent volume), while total extraction time can vary from 6 to 24 h depending upon the matrix and the solubility of the desired compounds. Conservative Soxhlet extraction has some advantages because the continued heating of the solvent keeps the temperature constant, avoids the filtration step and allows the extraction yield to be increased by parallel runs. It is possible to extract higher sample masses as compared with other modern extraction techniques, for example MAE and SFE (Luque de Castro and García-Ayuso, 1998). The major drawbacks include the total use of large quantities of solvent, longer extraction times, but above all, the possible degradation of heated labile compounds due to the continual heating of the extract in the solvent reservoir.

Modified Soxhlet extractors. As this extraction is still very much used and considered a benchmark for other extraction techniques, it has been modified to increase its efficiency and overcome its drawbacks. Some of these will be discussed here.

Automatic Soxhlet extraction or Soxtec. In this case, the extraction process consists of three steps. In the first step, the thimble with the sample is dipped in boiling solvent for 60 min, and then submitted to a normal Soxhlet extraction for 60 min, after which solvent evaporation and condensation take

place in the Soxtec apparatus, reducing the final volume of the extract to 1–2 mL (Dean, 2009). The main advantages of this technique are the rapid extraction and lower solvent usage. Nurthen *et al.* (1986) compared the yield of resin and rubber extracted from guayule using a Soxtec apparatus and a conventional Soxhlet, finding a 98% correlation between them.

Focused microwave-assisted Soxhlet extraction. In focused microwave-assisted Soxhlet extraction (FMASE), as the name indicates, energy is provided by microwaves rather than an electric heater or water bath, but the basic principal is almost same as conventional Soxhlet extraction. Microwave energy helps to break the sample matrix while solvent drips down onto the sample, is collected and recycled again. García-Ayuso *et al.* (1998) developed this technique and reported a reduction of extraction time from 8 h to 50–60 min. Priego-Capote and Luque de Castro (2005) also reported that by using FMASE, extraction time for total fat from bakery products decreased from 16 h to 60 min. They compared the results with the Association of Analytical Communities (AOAC) reference extraction method and found no significant difference in terms of recovery. Some other modifications are ultrasonic Soxhlet extraction and high-pressurised Soxhlet extraction that are extensively reviewed by Luque de Castro and García-Ayuso (1998).

Factors affecting Soxhlet extraction. As in any other extraction technique, some of the factors affecting the recovery, yield and quality of analytes are the time of extraction, temperature and sample size. In the case of a modified Soxhlet apparatus such as FMASE, the energy and duration of the radiation exposure can significantly affect the analyte extraction. Several optimisation studies have been done using different sample matrixes. Awolu *et al.* (2013) optimised the extraction for three factors, namely sample weight, particle size and extraction period using response-surface modelling, finding that all three significantly affected the yield of oil from *Azadirachta indica*. Similarly, the residual moisture content, time, solvent to mass ratio and particle size were also found to significantly affect the oil yield from *Dacryodes deulis* (Mampouya *et al.*, 2013).

In metabolomics. As compared with the other extraction methods mentioned above, Soxhlet extraction is considered to be the least suitable for metabolomics studies (Kim and Verpoorte, 2010), the major drawbacks being the longer extraction times and the continued heating of the extract. Although the longer extraction time may result in higher yields, it could also increase the possibility of the formation of artefacts (Maltese *et al.*, 2009). The possibility of solvent reactivity with analytes due to long exposure and high temperature should also be considered, a good example of which is the complete quaternisation of strychnine when using Soxhlet extraction on *Strychnos nux vomica* (Bisset and Choudhury, 1974). In spite of these drawbacks, Soxhlet extraction is still successfully applied to extract non-polar and weakly polar metabolites from sold matrixes (Schäfer, 1998; Iverson *et al.*, 2001). In some other studies, Soxhlet extraction accounted for the total phenolic content of mushrooms and different fruits (Cheung *et al.*, 2003; de Oliveira *et al.*, 2009). Thus, care should be taken, especially in case of metabolomics studies, to avoid the degradation of metabolites using the modified Soxhlet extraction method.

Comprehensive extraction method

Considering the different methods and solvents discussed above, it is quite clear that it might not be possible or even desirable to extract all metabolites in one single step. Analytical restrictions lead to the need to clean up extracts, because none of the available platforms can handle such a large amount of analytes due to interference issues, even if such an extract was possible to obtain. It is also clear that the ambition of finding a one-for-all extraction solvent or protocol that would be universally applicable is unrealistic, because differences in matrices, profiling aims and analytical platforms make this impossible.

Considering this, our group at the Natural Product Laboratory developed what we call the comprehensive extraction method. A detailed description of this method can be found in Yuliana *et al.* (2011). It is based on the use of a gradient of two or more solvents to increase the polarity range of the metabolite extraction. The ground plant material, together with an anti-clogging

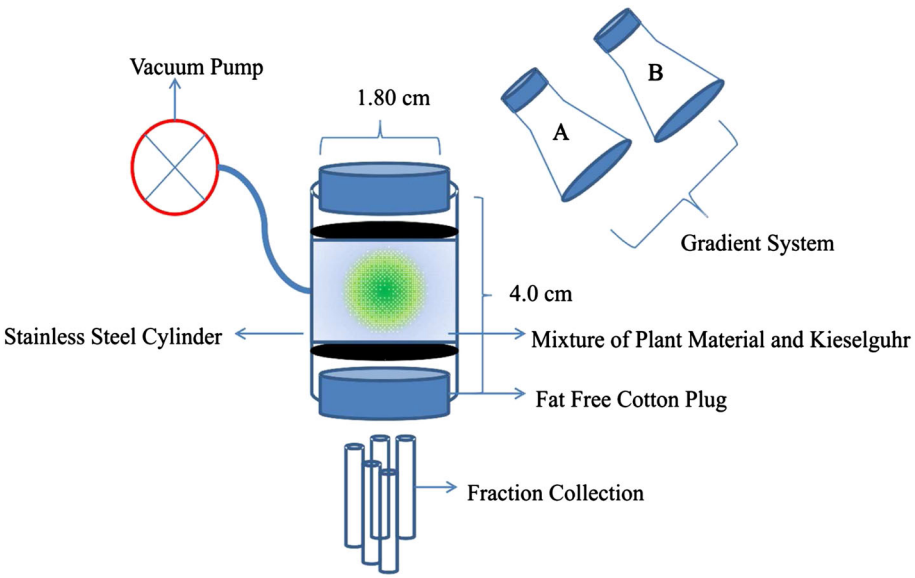

Figure 2. Graphical representation of the comprehensive extraction method.

agent, such as kieselguhr, is packed into a stainless steel column of 4.00 × 1.80 cm, which is then connected to a pump (Fig. 2). A gradient of increasing polarity of solvents is then pumped through the column and fractions are collected at predetermined time points. The greatest advantage of this method is the ability to extract a wide range of metabolites in conditions that preserve their integrity. In a conventional method it is quite possible to overlook the novel activity of a certain metabolite in a certain plant because the desired molecule did not have enough affinity with the extraction solvent and was thus not extracted. However, using a gradient solvent system and collecting fractions at different time points can minimise this risk. Thus, in principle, all metabolites should be extracted and the sum of metabolites obtained in all the fractions should represent the total metabolome (Yuliana *et al*., 2011). At the moment this method has been validated only for plants, but it could be successfully applied to other organisms.

From the literature discussed above, extraction is clearly a very important and critical step that is particularly challenging in metabolomics. Attention has to be paid not only to solvent–solute affinity, but also to possible solute–matrix interactions that can cause significant variations in collected data. Thus, the validation of proposed extraction methods or at least some evaluation of their extraction capacity is tedious, but inevitable. It involves testing profiles of extracts obtained with different solvent systems that are compatible with the analytical platform to be used and comparing their performance, bearing the goals of the studies to be undertaken in mind.

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