Chapter

Analytical Methods of Isolation and Identification

Weisheng Feng, Meng Li, Zhiyou Hao and Jingke Zhang

Abstract

The chemical constituents of plants are complicated, and monomeric compounds must be obtained via extraction and isolation before structure identification, bioactivity screening, and so on. In recent years, the new technologies and methods of the extraction, isolation, and structural identification have come forth, which promote the speed of extraction and analysis of phytochemicals. The chemical structures of compounds from plants must be identified or elucidated, which may provide the necessary basis for further study on the bioactivities, structure-activity relationships, metabolisms *in vivo*, structural modification, and synthesis of the active compounds. The amount of chemical constituents isolated from plants is often minor, so the structural studies are often difficult to carry out with classical methods. Therefore, spectral analysis is mainly used. This chapter describes the isolation and identification methods during the study of phytochemicals.

Keywords: extraction, isolation, structure elucidation, spectroscopic methods

1. Introduction

The phytochemicals rich in plants have shown to be beneficial for prevention of diseases as well as long-term health. Plants are generally consumed as sources of essential compounds such as saccharides, coumarins, lignans, flavonoids, terpenoids, and steroids. The health benefits and the composition from plant have been described more and more in the literature. Because of the complexity of plant chemical constituents, pure phytochemicals must to be obtained via extraction and isolation before structure identification, bioactivity screening, and so on. In recent years, new technologies and methods of extraction occurred, which accelerate the extraction and analysis of phytochemicals.

Extraction is the first step of phytochemistry research, which is also the necessary work before the isolation of effective constituents. The purpose of extraction is to get the objective chemical constituents to the utmost extent and avoid or reduce the solution of unwanted constituents.

The separation of phytochemicals is a process of isolating the constituents of plant extracts or effective parts one by one and purifying them into monomer compounds by physical and chemical methods. Classical isolation methods, including solvent extraction, precipitation, crystallization, fractional distillation, salting-out, and dialysis, are still used commonly at present. On the other hand, modern separation technologies such as column chromatography, high performance liquid chromatography, ultrafiltration, and high performance liquid drop countercurrent chromatography also play an important role in the separation of phytochemicals [1–3].

The chemical structures of plant compounds must be identified, which may provide the necessary basis for further study on the bioactivities, structure-activity relationships, metabolisms *in vivo*, structural modification, and synthesis of the active phytochemicals.

The structural studies are often difficult to carry out with classical chemical methods, such as chemical degradation and derivative synthesis, because of the minute amount of compound isolated from plants. Therefore, spectral analysis is mainly used. That is, consuming sample as little as possible to obtained structural information as much as possible by measuring and analyzing various spectra [4].

2. Extraction of phytochemicals

2.1 Solvent extraction methods

2.1.1 Principles

Solvent extraction is the commonest method to extract plant material. The main purpose is to select the suitable solvent to extract target plant materials efficiently. During the extraction, the solvent has to diffuse into the cell membrane in the first instance, in the following step it has to dissolve the solutes, then intracellular and extracellular concentration difference is formed, and finally it has to diffuse out of the cells enriched in the extracted solutes [5].

Selecting suitable solvents is the key of the solvent extraction method. Using a solvent of an appropriate polarity according to the principle of "like dissolves like" is the main point to select solvent. Thus, hydrophilic solvents are used to solubilize hydrophilic chemical constituents and vice versa. The hydrophilicity or lipophilicity of solvents and chemical constituents could be predicted by polarity. The plant compounds, such as terpenoids and steroids, possess low polarity, and could be dissolved into lipophilic solvents such as chloroform and ether, while chemical constituents, such as carbohydrates and amino acids, possess rather high polarity and could be dissolved into water and aqueous ethanol.

Solvents commonly used for extracting chemical constituents of plants are in the order of weak to strong polarity as follows: petroleum ether < carbon tetrachloride < benzene < dichloromethane < chloroform < ether < ethyl acetate < n-butanol < acetone < ethanol < methanol < water.

Water is a cheap, easy to get, and nontoxic solvent with strong polarity. It could be used to extract phytochemicals with strong polarity, such as inorganic salts, saccharides, amino acids, tannins, proteins, organic acid salts, alkaloid salts, and glycosides. Acid or alkaline water is applied sometimes to increase the solubility of certain specific components. Acid water could extract alkaline materials, such as alkaloids, via the formation of salts. Similarly, organic acids, anthraquinoids, flavonoids, coumarinoids, phenols, and other acidic materials could be extracted via the formation of salts. The disadvantage to extract chemical constituents with water is that the aqueous extract is easy to go moldy, so difficult to preserve. Additionally, water possesses high boiling point, and the water extract needs to be concentrated for a rather long time. Furthermore, the water extract contains many impurities such as proteins, pectins, tannins, mucilages, and inorganic salts, which make the extraction of target components difficult.

Hydrophilic organic solvents are strong-polarity and water miscible, such as methanol, ethanol, and acetone. Ethanol is the most commonly used hydrophilic organic solvent. Chemical constituents could be extracted by ethanol of different concentrations according to their properties. Furthermore, ethanol is inexpensive,

safe, and concentrated easily. Additionally, ethanol extract is not readily moldy and glycosides are hard to be hydrolyzed in ethanol extract. Thus, ethanol is one of the most commonly used solvents in laboratories and industrial production. Methanol possesses similar property to ethanol and lower boiling point. However, methanol has rather strong toxicity, so we have to pay attention to safety when it has to be used. Acetone is a good solvent to extract lipid-soluble chemical constituents. However, acetone is easy to volatilize and flame, and it possesses certain toxicity.

Petroleum ether, benzene, chloroform, ether, ethyl acetate, dichloroform, and so on are lipophilic organic solvents and are not miscible with water. They could be applied to extract lipophilic components, such as volatile oils, fats, chlorophyll, lactones, phytosterols, some alkaloids and some aglycones (aglycones of flavonoids, anthraquinoids, steroids, and so on). These solvents possess low boiling points and are easy to concentrate. However, strong-volatility, large loss, flammability, toxicity, and high price are their disadvantages. Additionally, they are difficult to permeate into plant cell tissues.

Solvent extraction methods could be classified as cold extraction and hot extraction roughly by whether heating or not.

2.1.2 Immersion method

It is a method to dissolve out phytochemicals with appropriate solvents at room or low temperatures (<80°C). It is suitable to extract phytochemicals easily to be destroyed at high temperature. The plants with abundant starches, pectins, gums, or mucilages could also be extracted with this method. Firstly, plant powder or pieces should be loaded in the adequate container, and then the suitable solvents (water, ethanol, aqueous ethanol, and so on) are added into it to immerse the material for the given length of time. Discontinuous stirring or shaking during the process could accelerate dissolution rate. The immersion method is simple but inefficient, and the extraction ratio is also low. Furthermore, aqueous extract is easy to go moldy, so addition of appropriate preservatives is necessary.

2.1.3 Percolation method

The coarse particles of plants should be loaded in percolation apparatus and immersed with suitable solvent for 24–48 h, then collect the percolates at the bottom of percolation apparatus. New solvent should be added at the top of percolation apparatus constantly during the percolation process. It possesses higher efficiency than the immersion method because of the sustained concentration difference during the process. However, this procedure is complex and consumes rather much solvent and long time.

2.1.4 Decoction method

Load short segments, thin pieces, or coarse powder into an appropriate container, add water, and heat it to boiling; the components are then extracted. It is easy to operate; most of the constituents could be extracted in various degrees. Nevertheless, rather much nontargeted components could also be extracted, and it is not suitable to the extraction of volatile compounds and thermal unstable compounds. Furthermore, it is not suitable to extract plants with lots of starches [6].

2.1.5 Refluxing method

It is a method to extract plant chemical constituents by organic solvent using heating and refluxing. Refluxing apparatus is necessary so as not to waste solvents,

and the toxicity to operators or ruin the environment is deduced. It is applicable to extraction of lipophilic phytochemicals, such as steroids, anthraquinoids, and terpenoids. It is an extraction method of high efficiency but complex, and consumes much more solvent. This method is not applicable to extract thermal unstable chemical constituents because of long time heating.

2.1.6 Constant refluxing method

It is a method developed based on the refluxing method. Soxhlet extractor is the most frequently used constant refluxing apparatus. This method avoids disadvantages of consuming too much solvent and complex operation. However, as a refluxing method, constant refluxing method is not applicable to extract thermally unstable compound either because of long time heating.

2.1.7 Supercritical fluid extraction method

In the supercritical state, the supercritical fluid is contacted with the plant tissues. By controlling different temperatures, pressures and different kinds and contents of entrainers, the supercritical fluid can selectively extract the components of different polarities, boiling points, and molecular weights successively. This method is called the supercritical fluid extraction (SFE) method [7].

The critical point of a pure substance is defined as the highest temperature and pressure at which the substance can exist in vapor-liquid equilibrium. At temperatures and pressures above this point, a single homogeneous fluid is formed, which is known as supercritical fluid (SF). SF is heavy like liquid and has low viscosity like gas meanwhile. SF possesses rather large diffusion coefficient and could dissolve many compounds well. A number of materials could be used as SFs, such as ammonia, ethane, difluoro-dichloromethane, heptane, and so on, while the most widely used SF is CO_2. The critical temperature of CO_2 (T_c = 31.26°C) is close to room temperature, and the critical pressure (P_c = 7.2 MPa) is not too high. CO_2 also has a series of other advantages, such as nontoxicity, odorless, nonflammable, chemical stability, and low cost, which allowed it to be the most commonly used solvent in SFE. CO_2 is a nonpolar substance and applicable to extract lipophilic compounds. However, its dissolvability is weak compared to strong polar substances. Hence, entrainers are always added to improve the solubility of SF CO_2 during the extraction of polar compounds. Entrainers, which are added into SF little, could enhance solubility of SF significantly. The commonly used entrainers are methanol, ethanol, water, acetone, ethyl acetate, acetonitrile, and so on.

The extraction of nonpolar and medium-polar components by SFE can avoid the sample loss and environmental pollution caused by solvent recovery in traditional extraction methods, especially for the extraction of volatile compounds with thermal instability.

The biggest advantage of SFE is that it can be performed at near-room temperature, and almost all the active ingredients in the product can be retained. There is no residual organic solvent in the process. The product has high purity and high yield. Additionally, the operation is simple and energy saving.

Compared with other conventional separation methods, SFE possesses the following advantages: (1) No residual organic solvents, fast extraction speed, simple process, high yield, and easy operation; (2) no flammable and explosive dangers, no environmental pollution. Low extraction temperature, suitable for the extraction of thermal unstable components; (3) the dissolution properties

of SF are easy to improve, only the pressure needs to be changed at a certain temperature; (4) entrainers can be added to change the polarity of the extraction medium to extract polar substances; extraction medium can be recycled with low cost; (5) it could be applied combined with other chromatographic techniques, such as GC, IR, GC–MS, and HPLC, to extract, separate, and determine phytochemicals efficiently and quickly, so as to achieve the integration of extraction and quality analysis. However, supercritical extraction has some limitations: strong solubility of fat-soluble components, weak solubility of water-soluble components, high cost of equipment, resulting in higher product costs, and cleaning equipment is difficult.

Supercritical fluid extraction (SFE) technology has achieved gratifying results in the fields of medicine, chemical, food, light industry, and environmental protection. Especially, it has been widely used in phytochemical extraction field, such as the extraction of alkaloids, volatile oils, phenylpropanoids, flavonoids, organic acids, glycosides, terpenoids, and so on.

2.1.8 Ultrasonic extraction method

It is a method of solvent extraction assisted by ultrasound. Ultrasonic wave is a kind of elastic mechanical vibration wave. The vibration frequency is as high as 20 KHz in elastic medium. The ultrasonic wave could vibrate the liquid medium. When the vibration is sparse, many small holes are formed in the medium. The instantaneous closure of these small holes can cause a pressure of up to thousands of atmospheric pressures. At the same time, the local temperature can rise to 1000°C. It can cause instantaneous rupture of the cell wall of plants and the whole organism, and make the solvent permeate into the cells of plants. This accelerates the dissolution of active ingredients in plants into solvents. Ultrasonic wave extraction could shorten the extraction time and improve the extraction efficiency, but could not change the structures of chemical constituents meanwhile.

Ultrasonic extraction technology has been widely used in the extraction of natural products in recent years, for example, extraction of soy isoflavones; see [8].

2.1.9 Microwave-assisted extraction method

Microwave refers to the electromagnetic wave whose wavelength is in the range of 0.1–100 cm (the corresponding frequency is 300–300,000 MHz), which is between infrared and radio waves. Polar molecules can absorb microwave energy, then release energy in the form of thermal energy, which makes the temperature inside the medium rise rapidly, causes the rather high pressure inside, and then the components flow out and dissolve in the solvent. On the other hand, the electromagnetic field produced by microwave can make some components diffuse to the interface of the extraction solvent, accelerating their thermal movement, which not only improves the extraction efficiency but also reduces the extraction temperature [9].

Microwave-assisted extraction has the advantages of less decomposition of chemical constituents, shorter time, lower energy consumption and less environmental pollution. Microwave-assisted extraction has been widely used in a series of fields of perfume, condiments, natural pigments, herbal medicine, cosmetics, soil and environmental analysis, and so on. In China, microwave-assisted extraction technology has been used in hundreds of Chinese herbal medicine extraction, such as *Pueraria lobata*, Panax notoginseng, Ginkgo, and so on, for example, the extraction of tea polyphenols and tea caffeine from green tea leaves; see [10].

2.2 Steam distillation method

Steam distillation is suitable for the extraction of volatile components which can be distilled with steam without being destroyed and are insoluble in water. These compounds' boiling points of are mostly higher than 100°C, and they possess certain vapor pressures at about 100°C. The principle of steam distillation is that the vapor pressure of each component is equal to that of their pure state, while the existence of another liquid does not affect their vapor pressure. The total vapor pressure of the mixing system is equal to the sum of the vapor pressures of the two components. Because the total vapor pressure of the system is higher than that of any single component, so the boiling point of the mixture is lower than that of any component. It is mainly used to extract volatile oils, some alkaloids, and phenolic substances of small molecules from plants.

2.3 Sublimation method

The process that solid material converts into steam directly without melting after heating is called sublimation. The phenomenon that steam condenses into solid after cooling is called deposition. Some natural chemicals have sublimation properties, which can be extracted directly with the sublimation method, for example, the extraction of camphor from camphor wood and caffeine from tea. In addition, some small molecular alkaloids, coumarins, organic acids, and other components also have sublimation properties, such as aesculetin and benzoic acid. However, it is easy to carbonize natural products because of long heating time. The volatile tar-like substances often adhere to sublimates, which are difficult to remove and often accompanied with thermal decomposition. The yield of this method is often low, and it is not suitable for large-scale production.

2.4 Pressing method

When the content of active ingredients is relatively high and exists in the juice of plants, the juice can be extracted directly from fresh raw materials. Volatile oils can also be extracted from plant tissues by mechanical pressing, such as orange peel oil and lemon oil. It is performed at room temperature, so its components will not be decomposed by heat. However, the products obtained are impure and often contain impurities such as water, mucoid substances, and cell tissues, so they are often turbid, and it is not easy to press the volatile oil in plants entirely. Therefore, the crushed residue is often distilled by steam to extract volatile oils completely. For example, the black soybean oil from black soybean is often extracted with the low-temperature pressing method.

3. Isolation and purification of phytochemicals

The separation of phytochemicals is a process of isolating the constituents of plant extracts or effective parts one by one and purifying them into monomer compounds by physical and chemical methods. Classical isolation methods, including solvent extraction, precipitation, crystallization, fractional distillation, salting-out, and dialysis, are still used commonly at present. On the other hand, modern separation technologies such as column chromatography, high performance liquid chromatography, ultrafiltration, and high performance liquid drop countercurrent chromatography also play an important role in the separation of phytochemicals. This section describes the common methods and their specific applications in isolation of phytochemicals.

3.1 Solvent method

3.1.1 Acid and basic solvent method

It is carried out according to the different acidity and alkalinity of each component in the mixture. Water-insoluble alkaline organic components, such as alkaloids, could react with inorganic acids and form salts, which can be separated from nonalkaline and water-insoluble components. Acid components with carboxyl or phenolic hydroxyl groups can be salted by bases and dissolved in water. Components with lactone or lactam substructures can be saponified and dissolved in water and then isolated from other water-insoluble components. The total extract can be dissolved in lipophilic organic solvents (ethyl acetate is commonly used) and extracted respectively with acid water and alkali water, and then the total extract would be divided into acidic, alkaline, and neutral parts. Of course, the total extract can also be dissolved in water and extracted with organic solvents after adjusting the pH value. The alkalinity or acidity of the fractions are different and can be separated further by pH gradient extraction.

When using the acid and basic solvent method, attention should be paid to the strength of acidity or alkalinity, the contact time with the separated components, heating temperature, and time, so as to avoid the structural changes of some compounds under severe conditions or the chemical structures cannot be restored to the original states.

3.1.2 Polarity gradient extraction method

This method is to achieve the separation aim based on the different polarity of each component in plant extracts and the different partition coefficients in two-phase solvents. Generally, different two-phase solvent systems are selected according to the polarity of components in plant extracts. For example, the components with strong polarity can be separated by n-butanol-water system, the components with medium polarity can be separated by ethyl acetate-water system, and the components with weak polarity can be separated by chloroform (or ether)-water system. During the operation, the plant extract should be dissolved by water firstly, and then the solution or suspension is extracted in a separating funnel with different organic solvent which is not miscible with water based on the polarity difference. Usually, the extract was extracted with petroleum ether (or cyclohexane) firstly, then ethyl acetate (or chloroform), and finally with water saturated n-butanol, as shown in **Figure 1**. Petroleum ether layer contains lipid-soluble compounds with low polarity. Ethyl acetate layer contains medium polar compounds such as monoglycosides, flavonoids, and compounds with more polar functional groups. N-butanol layer contains compounds with strong polarity, such as oligoglycosides and other water-soluble components. Compounds in water layer possess strongest polarity, such as glycosides with more glycosyl groups, carbohydrates, amino acids, proteins, and other water-soluble compounds.

3.2 Precipitation method

It is a method based on the formation of precipitation of some phytochemicals by reaction with specific reagents, or the precipitation of some components from the solution by adding specific reagents, which can reduce the solubility of some components in the solution. The precipitation reaction must be reversible if the target components are required to form precipitation. While if the components are nontarget, the precipitation generated will be removed, so the precipitation reaction can be irreversible. According the addition of reagents or solvents, this method could be classified as follows [11].

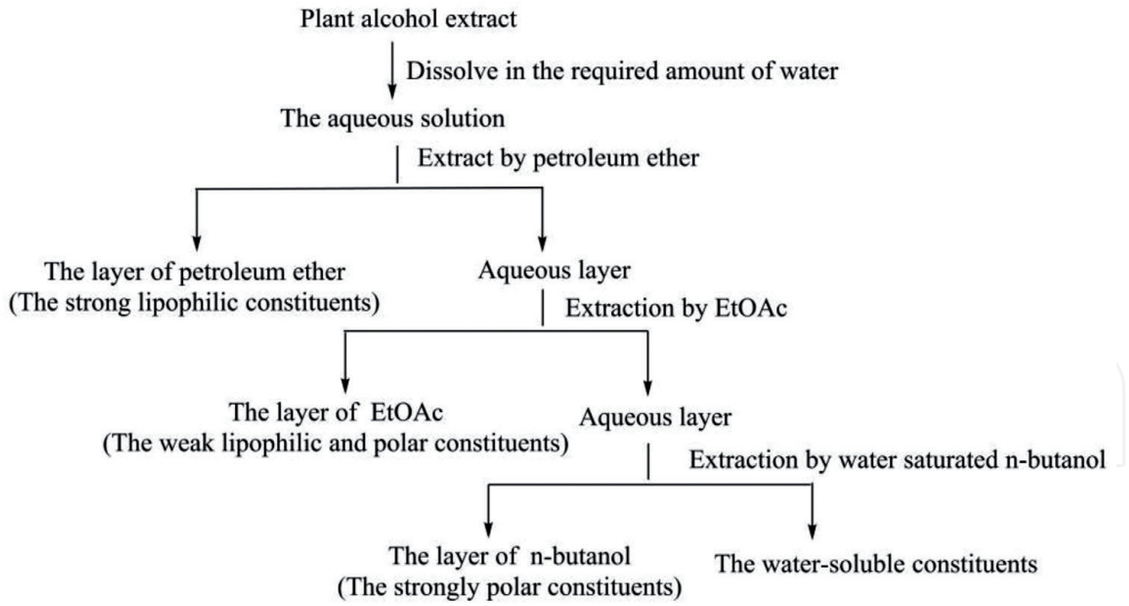

Figure 1.
Flow charts of common polarity gradient extraction method.

3.2.1 Solvent precipitation method

The solubility of some components in the mixed component solution can be changed by adding a specific solvent that can be mutually soluble with the solution, so it can be precipitated from the solution. The gradual precipitation by changing the polarity or amount of solvent added is called fractional precipitation. For example, using water as an extracting solvent to extract phytochemicals, ethanol is added to the water extracting concentrate to make its alcohol content more than 80%, and then polysaccharides, proteins, starch, gum, and so on will be precipitated and removed after filtration. The preceding procedure is called water extraction and ethanol precipitation. Crude polysaccharides from plants are often separated with this method. For example, see [12].

3.2.2 Exclusive reagent precipitation method

Some reagents could react selectively with certain chemical constituents to produce reversible precipitation, and the separation aims are achieved, which is called the exclusive reagent precipitation method. For example, alkaloid precipitation reagents such as Reynolds ammonium salt can precipitate after reacting with alkaloids, which can be used to separate alkaloids and nonalkaloids, or water-soluble alkaloids and other alkaloids. As another example, reactions of cholesterol and sterol saponins could form precipitation, which can separate them from triterpene saponins. Additionally, gelatin can precipitate tannins, which can be used to separate or remove tannins. In practical application, appropriate precipitation reagents should be selected according to the properties of target constituents and impurities in plants.

3.2.3 Salting out method

Adding inorganic salts to a certain concentration or saturated state in the water extract of plants can reduce the solubility of some components in water, thus they could be separated from water-soluble compounds. The inorganic salts commonly used for salting out are sodium chloride, sodium sulfate, magnesium sulfate, ferric

sulfate, etc. For example, extractions of tetrandrine from *Daemonorops margaritae* and berberine from *Berberis poiretii* could be achieved by salting out with sodium chloride or ammonium sulfate. Some water-soluble substances, such as proto-anemone, ephedrine, and matrine, are often extracted with organic solvents after adding a certain amount of salt to the water extract. For example, see [13].

3.3 Dialysis method

It is a method to let substances selectively penetrate through natural or synthetic semi-permeable membranes (or dialysis bags) under the action of concentration difference, pressure difference, or potential difference, so as to achieve the purpose of separation, classification, purification, or concentration. For example, when saponins, proteins, polypeptides, polysaccharides, and other substances in plants are separated and purified, dialysis can be used to remove inorganic salts, monosaccharides, and other impurities. On the contrary, large molecular impurities can also be left in the semi-permeable membrane, while small molecular substances can be separated and purified through the semi-permeable membrane into the solution outside the membrane [14].

3.4 Fractional distillation method

Fractional distillation is a method of separating components in liquid mixtures based on their different boiling points. It is usually categorized into atmospheric, vacuum, molecular distillation, and so on. It is mainly used for the separation of volatile oils and some liquid alkaloids in plants. For example, the boiling points of the two alkaloids in total alkaloids of *Cicuta virosa,* coniine, and conhydrine are 166–167°C for the former and 226°C for the latter, which are quite different from each other, and then they can be separated by the fractional distillation method. Generally, if the boiling point difference of compounds in liquid mixtures is above 100°C, the separation can be achieved by repeated distillation of the solution. If the boiling point difference of compounds is below 25°C, the fractionation column is needed. The smaller the boiling point difference is, the finer the fractionation device is needed [15].

3.5 Crystallization method

Crystallization is the process of solute precipitation from mother liquor with complex components, and it is an effective method to prepare pure substances. The initial crystallization is often impure and needs to crystallize again, which is called recrystallization. It is a method to separate compounds from the mixture by using the difference of solubility of each component in the solvent. Crystallization is one of the important technologies for plant chemists to prepare pure compounds.

When the content of a phytochemical is very high in one plant, crystals can be obtained by cooling or slightly concentrating the extract after extraction with appropriate solvent. For example, see [16].

Selecting suitable crystallization solvent is the key of the crystallization method. The ideal solvents for crystallization should possess the following characteristics: high solubility for the components to be purified at high temperature, low solubility at low temperature, insoluble for the impurities at high and low temperature, or soluble for the impurities at high and low temperature, moderate boiling point, no chemical reaction with the components to be crystallized, safe, low price, easy to obtain, and so on. Solvents commonly used for crystallization are methanol, ethanol, acetone, ethyl acetate, acetic acid, pyridine, etc. When crystals cannot be obtained with a single solvent, the crystallization operation can be carried out with a mixture of two or more solvents. Mixed solvents generally consist of two miscible

solvents, one of which has high solubility for the component to be crystallized, and the other has low solubility. Firstly, the sample to be crystallized is heated and dissolved in as few solvents as possible with high solubility. Then the second solvent with low solubility is added to the hot solution to make it turbid. Then the first solvent is added to dissolve the sample. The solution reaches saturation at this point and crystallizes when it is cooled. The purity of crystallization can be preliminarily identified by the crystal form, color, melting point, melting range, thin layer chromatography, paper chromatography, etc.

3.6 Classical chromatographic methods

Chromatography is the most commonly used method for the separation of chemical constituents of natural products. It possesses advantages of high separation efficiency, rapidity, and simplicity. By choosing different separation principles, different operation modes, different chromatographic packings, or applying various chromatographic methods jointly, the separation and purification of various types of phytochemicals could be achieved. It can also be used for the identification of compounds.

3.6.1 Adsorption chromatography

It is a kind of chromatography based on the difference of adsorptive capacity of adsorbents to different compounds. The commonly used adsorbents include silica gel, alumina, activated carbon, polyamide, and so on. Silica gel adsorption chromatography is widely used, and it is suitable to the separation of most of the plant chemical constituents. Alumina adsorption chromatography is mainly used for the separation of alkaline or neutral lipophilic components, such as alkaloids, steroids, and terpenoids. Activated carbon is mainly used for the separation of water-soluble substances, such as amino acids, carbohydrates and some kinds of glycosides. Polyamide, which allows the separation to take place based on the formation of kinds of hydrogen bonds, is mainly used for the separation of phenols, quinones, flavonoids, anthraquinones, tannins, etc. [17].

3.6.2 Gel chromatography (exclusion chromatography, molecular sieve chromatography)

Molecular sieve is the main principle of gel chromatography, which can separate mixture compounds according to the pore size of the gel and the molecular size of the compounds. Gel is a kind of solid material with a porous network structure. The molecules of the separated substances are different in size, so their ability to enter the gel is different. When the mixture solution passes through the gel column, the molecules smaller than the gel pores can enter the gel interior freely, while the molecules with larger size than the gel pores cannot enter the gel, and only pass through the gel particle gaps. Therefore, different movement rates are emerged. The molecules with large sizes are not excluded, and the retention time is shorter. The molecules with small sizes are detained because of its diffusion into the pores, thus the retention time is longer. There are many kinds of commercial gels, dextran gel and hydroxypropyl dextran gel are used most commonly [18].

3.6.3 Ion exchange chromatography

It is to separate chemical constituents according to the difference of dissociation degrees. In this method, ion exchange resin is applied as stationary phase and water or solvent mixed with water as mobile phase. The ionic components existing in the

mobile phase are absorbed by ion exchange resin after ion exchange reaction. Ion exchange chromatography is suitable for the separation of ionic compounds, such as alkaloids, amino acids, organic acids, peptides, and flavonoids. The ability of ion exchange reaction between compounds and ion exchange resins mainly depends on the compounds' dissociation degree and the amount of electric charges. If the dissociation degree of a compound is high (acidic or alkaline), it is easily exchanged on resins and difficult to elute. Therefore, when the compounds with different degree of dissociation are exchanged on the resin, the compounds with lower degree of dissociation are eluted before those with higher degree of dissociation [19].

3.6.4 Macroporous adsorption resin chromatography

It is a chromatographic method which combines the principle of adsorption and molecular sieve. Its chromatographic behavior possesses reversed-phase properties. Macroporous resin is a kind of solid macromolecule material with no dissociable group and porous structure and is insoluble in water. It is widely used in the separation and enrichment of natural compounds because of its stable physical and chemical properties (insoluble in acids, bases, and organic solvents).

In practical work, the water solution of the mixture to be separated is usually washed by water, water-containing alcohol solution with low to high concentration. The mixture can be separated into several components. The regeneration of macroporous adsorbent resin is convenient. It is often washed by 1 mol/L hydrochloric acid and 1 mol/L sodium hydroxide solution, respectively, first, then washed by distilled water to neutral, and stored in methanol or ethanol. The alcohol should be washed out with distilled water before using.

3.6.5 Partition chromatography

It is a kind of chromatography method to separate components by using different partition coefficients between stationary phase and mobile phase, which are immiscible liquids. Partition chromatography could be divided into normal phase chromatography and reverse phase chromatography. The polarity of stationary phase is stronger than that of mobile phase in normal phase partition chromatography, which is mainly used to separate polar and moderately polar molecular compounds. Carriers commonly used in normal phase distribution chromatography include silica gel, diatomite, cellulose powder, etc. Silica gel with water content of more than 17% can be used as a carrier for partition chromatography because of its loss of adsorption. It is the most widely used carrier for partition chromatography. In reverse phase partition chromatography, the polarity of mobile phase is stronger than that of stationary phase. The commonly used stationary phase is octadecylsilylated silica (ODS). The mobile phase is usually methanol-water or acetonitrile-water system, which is mainly used for the separation of nonpolar and moderately polar molecular compounds.

3.7 New technologies and methods

3.7.1 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a rapid separation and analysis technology developed on the basis of conventional column chromatography. Its separation principle is the same as regular column chromatography, including adsorption chromatography, gel chromatography, partition chromatography, ion exchange chromatography, and other methods. HPLC columns are produced

with particle fillers (particle diameter 5–20 μm) and high pressure homogenate column loading technology. The eluents are pressed into the column by a high pressure infusion pump and equipped with high sensitive detectors and automatic recording and collection devices. As a result, it is far superior to conventional column chromatography in separation speed and efficiency. It has the characteristics of high efficiency, high speed, and automation. Preparative HPLC can be used to prepare a large amount of samples of high purity. HPLC has played an increasingly important role in the separation, qualitative identification, and quantitative analysis of plant chemical constituents. During the separation of many plant chemical constituents, it is necessary to separate trace constituents from a large amount of crude extracts. Usually, in the final stage of separation, samples with high purity are prepared by high or medium pressure liquid chromatography. Constant concentration eluents are mostly used in preparative HPLC. However, gradient elution is sometimes applied for samples that are difficult to be separated. Moreover, HPLC retains the advantages of liquid chromatography, such as a wide range of application and flexibility of mobile phase change. It can be applied to chemical constituents of difficult gasification, high molecular weight, or thermal instability.

The detectors commonly used in HPLC are ultraviolet detectors and differential refractive index detectors, but both have limitations. Differential refractive index detectors are sensitive to temperature change, the detection of a small amount of substances is often not ideal, and gradient elution cannot be used. As for ultraviolet detectors, they cannot detect samples without ultraviolet absorption. In recent years, a kind of mass detector, called evaporative light scattering detector (ELSD), has been applied in HPLC. It can not only detect samples without ultraviolet absorption, but also use gradient elution. It is suitable for most nonvolatile components [20].

3.7.2 Droplet counter-current chromatography (DCCC)

DCCC is an improved liquid-liquid partition chromatography based on the counter-current partition method. The formation of droplets is required when the mobile phase passes through a liquid stationary phase column. Droplets of mobile phase contact with stationary phase effectively, and form new surfaces in thin partition extraction tubes constantly, which promote the partition of solutes in two-phase solvents, and the chemical components of mixtures are isolated in immiscible two-phase droplets due to different partition coefficients. This method is suitable for the separation of phytochemicals with strong polarity. The separation effect is usually better than counter-current partition chromatography, and there is no emulsification phenomenon. Furthermore, nitrogen is used to drive the mobile phase, so the separated substance will not be oxidized by oxygen in the atmosphere. However, the solvent system which can generate droplets must be selected in this method, the amount of sample treated is small, and special equipment is needed.

DCCC possesses good reproducibility, and can handle crude extract samples of milligram to gram grade. It can be used in either acidic or basic conditions. Because no solid separation carriers are used, the phenomenon of irreversible adsorption and band broadening of chromatographic peaks can be avoided. Compared with preparative HPLC, DCCC consumes less solvent, but the separation time is longer and the resolution is lower. For example, see [21].

3.7.3 High speed counter-current chromatography (HSCCC)

HSCCC is also a liquid-liquid partition chromatography. It is another mild form of chromatography with no solid support and hence no chance of loss of substrate by binding to the column. The only media encountered by the sample are solvent

and Teflon tubing. The former is common to all forms of chromatography and the latter to most. The chemical constituents with higher partition coefficient in mobile phase are eluted first, whereas those with higher partition coefficient in stationary phase are eluted later.

HSCCC chromatography could avoid the shortcomings of irreversible adsorption and abnormal tailing of chromatographic peaks caused by solid carriers in liquid chromatography because it does not need solid carriers. The sample recovery is near 100% from a chromatography. It also has advantages of good reproducibility, high purity of separated compounds, and fast speed. It is suitable for the isolation and purification of wide kinds of phytochemicals, such as saponins, alkaloids, flavonoids, anthraquinoids, lignans, triterpenes, proteins, and carbohydrates. For example, see [22].

3.7.4 High performance capillary electrophoresis (HPCE)

It is an instrumental analysis method developed in the late 1980s combining classical electrophoresis with modern microcolumn separation technologies. In pharmaceutical analysis, the most commonly used separation modes are capillary zone electrophoresis, micellar electrokinetic capillary chromatography, and capillary gel electrophoresis. It is an efficient separation technology of large and small molecules in a hollow and thin inner diameter capillary (10–200 μm). The two ends of the capillary are immersed in a buffer solution and electrodes connected with a high voltage power supply are inserted separately. The voltage makes samples migrate along the capillary. According to the charge and volume of the separated substances, various molecules are separated under high voltage. In zone capillary electrophoresis, separation could be achieved by the movement of electrophoresis and electroosmotic flow. The strength of electroosmotic flow depends on the strength of electric field, PH value of electrolyte, composition of buffer solution, ionic strength, internal friction, and so on. Sample injection could be accomplished by pressing the sample into a capillary tube by atmospheric pressure or voltage.

HPCE has the advantages of high efficiency, microamount, economy, high automation, and wide application. However, it has the disadvantages of poor preparation ability, low sensitivity, and poor separation reproducibility. For example, see [23].

3.7.5 Affinity chromatography (AC)

Affinity chromatography is a unique chromatographic separation method based on the principle of reversible combination of high affinity and specificity between molecules. By simulating the reversible and specific interaction between biological molecules, affinity chromatography uses the adsorption medium coupled with affinity ligands as the stationary phase to adsorb target compounds. It is a development of adsorption chromatography. This method can selectively separate and analyze specific chemical constituents from complex samples. Firstly, ligands that can specifically bind to the target compounds are fixed on the filler carrier to make the chromatographic column. Then the mixture containing the target compounds is passed through the column. Only the target compounds which show affinity with the ligands can bind to the ligands and remain in the column. Finally, the adsorbed target compounds are eluted by changing the composition of the mobile phase and are separated from other chemical constituents. AC is mainly used for the separation and purification of proteins, especially enzymes, antigens, and antibodies. Its application range has been expanding along with the continuous development of technology in recent years. For example, see [24].

4. Structural identification of phytochemicals

The chemical structures of plant compounds must be identified or elucidated, which may provide the necessary basis for further study on the bioactivities, structure-activity relationships, metabolisms in vivo, structural modification, and synthesis of the active phytochemicals.

The quality of physiological active substances isolated from plants is often small, sometimes only a few milligrams, and the structural studies are often difficult to carry out with classical chemical methods, such as chemical degradation, derivative synthesis, etc. Therefore, spectral analysis is mainly used, that is, consuming sample as little as possible to obtain structural information as much as possible by measuring various spectra. Then comprehensive analysis is carried out with the assistance of literature data. If necessary, chemical means would be integrated into the former methods to determine the planar- and even the stereo-structures of the compounds.

4.1 Determination of the purity of the compounds

Before the structural investigation of an active compound, the purity must be determined, which is a prerequisite for the structural identification.

4.1.1 Measurement of physical properties

The crystals of each compound have certain shape, color, and melting point, which can be used as the basis for the preliminary determination of the purity. Generally, the crystal shape of a specific compound under the same solvent is consistent, the color is pure, and has a short melting range (generally at 1~2°C). But for compounds with double melting points or amorphous substances, the purity cannot be determined by this method.

4.1.2 Thin layer chromatography (TLC)

TLC, such as silica gel and paper chromatography, is the most commonly used method to determine the purity of compounds. Generally, a specific sample, showing an only spot (Rf value at 0.2~0.8) in three different developing agents, could be considered as a pure compound. In some cases, both normal and reverse phase chromatographic methods are needed.

4.1.3 Gas chromatography (GC) and high performance liquid chromatography (HPLC)

GC and HPLC are important methods in the purity determination of phytochemicals. GC is widely used in the analysis of volatile compounds. Both volatile and nonvolatile substances could be analyzed with HPLC, which possesses various advantages of high speed, high efficiency, sensitivity, and accuracy.

4.2 Major procedures of structural determination

The general procedures of structural determination of phytochemicals are shown roughly in **Figure 2**.

The structural identification of phytochemicals can be greatly simplified according to the researchers' habits, experiences, and skill levels of different technologies. However, the literature search almost runs through the whole process of structural

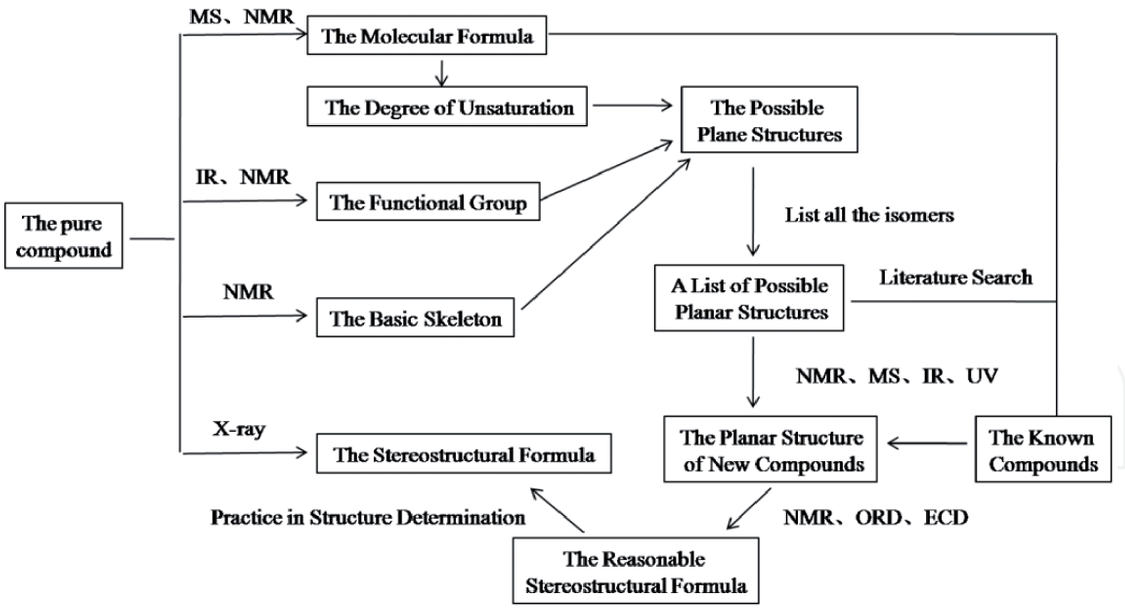

Figure 2.
The main procedures for studying the structures of phytochemicals.

research, no matter for known or new compounds. A large number of facts have been proved that taxonomically related plants, that is to say, plants of same or similar genus often contain chemical constituents of similar or even same chemical structures. Therefore, it is necessary to investigate literatures of chemical studies of the study object and the plants of its same and similar genera. It is necessary to understand not only the components from different plants of similar genera, but also their extraction methods, physicochemical properties, spectral data, and biosynthesis pathways before the extraction and separation of one specific plant. The SciFinder Scholar database is used most widely to quickly determine whether the compound was "known" or "unknown".

4.3 Spectral technologies

At present, spectrum analyses have become the main means to determine the chemical structures of plant chemicals. Particularly, with the developing of the superconducting nuclear magnetic resonance (NMR) and mass spectroscopic (MS) technologies, the speed of structural determination is greatly accelerated and the accuracy is improved. Here, the applications of infrared (IR), ultraviolet (UV), nuclear magnetic resonance (NMR), and mass (MS) spectra in the structural identification of phytochemicals are introduced briefly.

4.3.1 Ultraviolet-visible spectra (UV-Vis)

UV-vis spectrum is a kind of electron transition spectrum, which is generated after the molecules absorbing the electromagnetic waves with wavelength at the range of 200–800 nm. The valence electrons in the molecules absorb light of certain wavelengths and jump to the excited state from the ground state, and then UV spectra are recorded.

Compounds containing conjugated double bonds, α,β-unsaturated carbonyl groups (aldehydes, ketones, acids, and esters), and aromatic compounds could show strong absorption in UV spectra because of $n \rightarrow \pi^*$ or $\pi \rightarrow \pi^*$ transitions. Therefore, UV spectrum is mainly used to identify the presence of conjugated systems in the structures.

UV spectra could provide the following information: (1) the compounds show no UV absorption at 220–800 nm, indicating the compounds were aliphatic hydrocarbons, aliphatic cyclic hydrocarbons, or their simple derivatives. (2) The compounds show strong absorption at 220–250 nm, indicating that the compounds possess conjugated diene, α,β-unsaturated aldehyde, or ketone substructures. (3) The absorption at 250–290 nm is moderately strong, indicating that the compounds possess benzene rings or aromatic heterocycles. (4) Weak absorption at 250–350 nm indicates the presence of carbonyl or conjugated carbonyl groups. (5) Strong absorptions at above 300 nm indicate that the structures possess long conjugated chains.

Generally, UV spectrum can only provide part of the structural information, rather than the whole structural information of a compound, so it can only be used as an auxiliary method to identify the structures. It possesses practical value to determine the structures of phytochemicals with conjugated substructures.

4.3.2 Infrared spectra (IR)

IR is caused by the vibration-rotational energy level transition of the molecule, ranging from 4000 to 625 cm^{-1}. The region above 1250 cm^{-1} is functional group region, and the absorption of characteristic functional groups such as hydroxyl, amino, carbonyl, and aromatic rings occurs in this region. The region of 1250 to 625 cm^{-1} is fingerprint region, and the peaks appear mainly due to the stretching vibrations of C-X (X = C, O, N) single bonds, and various bending vibrations. IR is mainly used for the determination of functional groups and the types of aromatic ring substitution. In some cases, IR can also be used to determine the configuration of plant chemical constituents. For example, there is a significant difference between 960 and 900 cm^{-1} for 25R and 25S spirostanol saponins.

4.3.3 Mass spectrometry (MS)

In a mass spectrometer, mass and strength information of molecular and fragment ions is recorded after the molecules are ionized and enter into the collector under the action of electric and magnetic fields. The abscissa represents the mass-to-charge ratio (m/z) and the ordinate represents the relative intensity in a MS spectrum. Unlike IR, UV, and NMR spectra, MS is mass spectrum, which characterizes fragment ions, not an absorption spectrum. Its role is to determine weights, formulas, and fragment structures of molecules.

With the rapid development of modern techniques, new ion sources have emerged in recent years, which make MS play more important role in determining the molecular weights, elemental composition, detecting functional groups by cleavage fragments, identifying compound types, and determining carbon skeletons [25]. In the structural analysis, the information of molecular weights could be obtained on the basis of molecular ion peaks, and the molecular formula could be obtained by high-resolution mass spectrometry (HR-MS). Fragment ion peaks, combined with molecular ion peak, could be applied to conjecture chemical structures. Tandem mass spectrometry even can isolate and analyze the mixed ions again. According to the types of ion sources, common mass spectrometry could classified as electron impact mass spectrometry (EI-MS), chemical ionization mass spectrometry (CI-MS), field desorption mass spectrometry (FD-MS), fast atom bombardment mass spectrometry (FAB-MS), matrix-assisted laser desorption mass spectrometry (MALDI-MS), electrospray ionization mass spectrometry (ESI-MS), tandem mass spectrometry (MS–MS), and so on.

4.3.4 Nuclear magnetic resonance (NMR)

With the birth of Fourier transform spectrometer, the great progress of radionuclide research such as ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P, and the advancement of two-dimensional and three-dimensional nuclear magnetic technology, NMR has become the most important spectroscopic method to determine chemical structures. Particularly, hydrogen spectrum and carbon spectrum are most widely used. During the operation of nuclear magnetic resonance spectrometer, compound molecules are irradiated by electromagnetic waves in a magnetic field, energy level transitions occur after the atomic nuclei with magnetic distance absorb a certain amount of energy, and then NMR spectrum is obtained by mapping the absorption strength with the frequencies of the absorption peaks. It can provide structural information about the type and number of hydrogen and carbon atoms in the molecule, the modes they are connected, the surrounding chemical environment, configuration, and conformation [26].

4.3.4.1 Commonly used deuterated reagents

Samples used to measure NMR spectra include solids, liquids, and gases. Liquid high-resolution NMR is most widely used. The solvent used in the measurement of NMR must be deuterated. The commonly used deuterated reagents to dissolve samples and their chemical shifts of their residual proton and carbon signals are shown in **Table 1**.

4.3.4.2 Proton nuclear magnetic resonance spectroscopy (1H-NMR)

Resonance absorption peaks are generated after hydrogen protons absorb electromagnetic waves of different frequencies in an external magnetic field. ^{1}H-NMR possesses high sensitivity, easy measurement, and wide application. ^{1}H-NMR spectrum can provide structural information of chemical shifts (δ), coupling constants (J) that indicate the coupling relationships between different hydrogen nucleus, and the number of protons (the peak area is proportional to the number of protons that cause the absorption).

Because of the different surrounding chemical environment, the ^{1}H nuclei possess different magnetic cloud densities and magnetic shielding effects caused by the rotation around the nucleus, and then different types of ^{1}H nuclear resonance signals appear in different regions. Tetramethylsilane (TMS) is usually used as a reference compound. Compared with the general compounds, the shielding effect of protons and carbons on the methyl groups is stronger in TMS. Therefore, regardless of the hydrogen spectrum

Solvent	δ_C	δ_H
$CDCl_3$	77.0	7.24
CD_2Cl_2	53.8	5.32
CD_3OD	49.0	3.3
Acetone-d_6	29.8, 206.0	2.04
D_2O	—	4.7
DMSO-d_6	39.5	2.49
C_6D_6	128.0	7.16
C_5D_5N	123.6135.6149.9	7.2, 7.6, 8.7

Table 1.
Chemical shifts of common deuterated solvents (TMS is an internal standard).

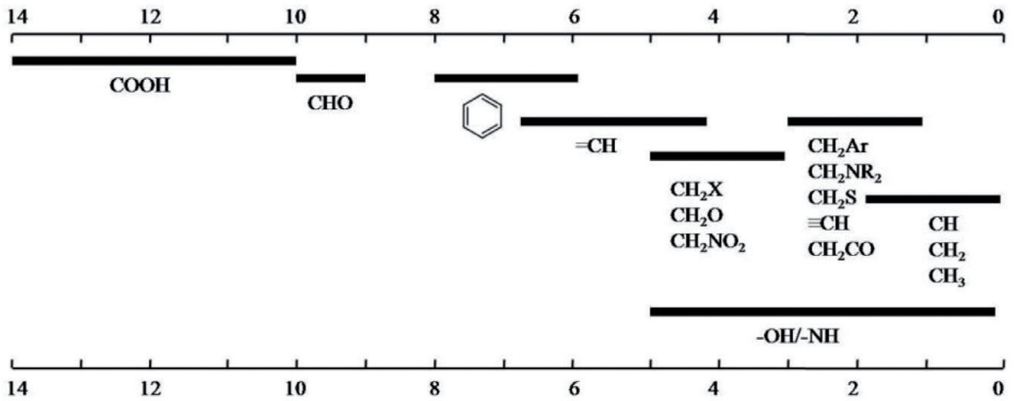

Figure 3.
^{1}H-NMR chemical shift range of common hydrogen protons.

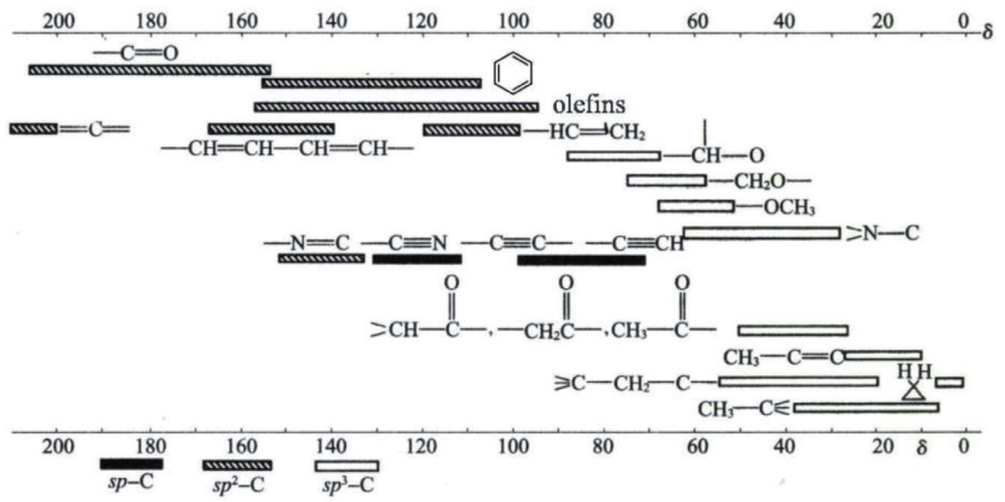

Figure 4.
^{13}C-NMR chemical shifts of common carbon signals.

or the carbon spectrum, the absorption peaks generated by the general compounds appear in the lower field than TMS, that is to say, δ values generated by common compounds is positive. The chemical shifts of the ^{1}H-NMR spectrum is mostly in the range of δ0–20. Some typical chemical shifts of ^{1}H nuclei are shown in **Figure 3** [4].

In addition to the normal ^{1}H-NMR spectrum technique, there are some auxiliary techniques that assist in structural analysis, such as selective decoupling, heavy hydrogen exchange, addition of reaction reagents, and dual irradiations.

4.3.4.3 Carbon nuclear magnetic resonance spectroscopy (^{13}C-NMR)

^{13}C-NMR spectra can provide structural information of organic compounds, including the number, types, and chemical environment of carbon atoms [27]. It is one of the important means for the structural identification of organic compounds. Especially, where there are serious signal peak overlaps in the ^{1}H-NMR spectrum, or the molecules contain several quaternary carbon atoms, ^{13}C-NMR spectra will provide crucial information for the structure identification. The chemical shifts of common carbon signals are shown in **Figure 4** [4].

Common ^{13}C-NMR techniques include proton broadband decoupling, off resonance decoupling (OFR), insensitive nuclei enhanced by polarization transfer (INEPT), and distortionless enhancement by polarization transfer (DEPT). Proton broadband decoupling and DEPT spectra are most commonly used at present.

4.3.4.3.1 Proton broadband decoupling

Proton broadband decoupling spectrum is measured after ^{1}H nuclei are saturated with broadband electromagnetic radiation. At this point, the couplings between ^{1}H and ^{13}C are completely eliminated, and all ^{13}C signals are shown as singlets, so it is very convenient to determine the chemical shift of ^{13}C signals. In addition, because of the NOE effect of ^{1}H after irradiation, the signal of ^{13}C signal connected with ^{1}H will be increased, while the quarterly carbon signal will show weak absorption peaks.

4.3.4.3.2 Distortionless enhancement by polarization transfer (DEPT)

It is an improved method of INEPT, in which a *J*-modulation is accompanied by a polarization transfer from the protons to coupled carbons, leading to significant improvement in sensitivity. In DEPT spectrum, by changing the pulse width (θ), which could be designed as 45°, 90°, and 135°, during irradiation of ^{1}H, different carbons could show different strengths and signs. The results are similar with INEPT spectrum. When θ = 45°, all CH, CH_2, and CH_3 groups display positive signals; when θ = 45°, only CH groups show positive signals; when θ = 135°, both CH and CH_3 groups show positive signals, while CH_2 groups show negative signals. Quarterly carbons show no signal peaks in DEPT spectra. An example of DEPT spectra is shown in **Figure 5**.

4.3.4.4 Two-dimensional nuclear magnetic resonance spectroscopy (2D-NMR)

Two-dimensional correlation spectroscopy (2D-COSY) is the most important and widely used in 2D-NMR spectroscopy. 2D-COSY spectra can be divided into homonuclear and heteronuclear correlation spectra. Both abscissa and ordinate represent chemical shifts in 2D-COSY. Common correlation spectrum types are show as follows.

4.3.4.4.1 ^{1}H-^{1}H COSY spectrum

It is a kind of chemical shift correlation spectrum between ^{1}H and ^{1}H. It is the coupling correlation spectrum between protons in the same coupling system. The adjacent hydrogen groups could be determined by their coupling relationships (^{3}J) shown in ^{1}H-^{1}H COSY spectra.

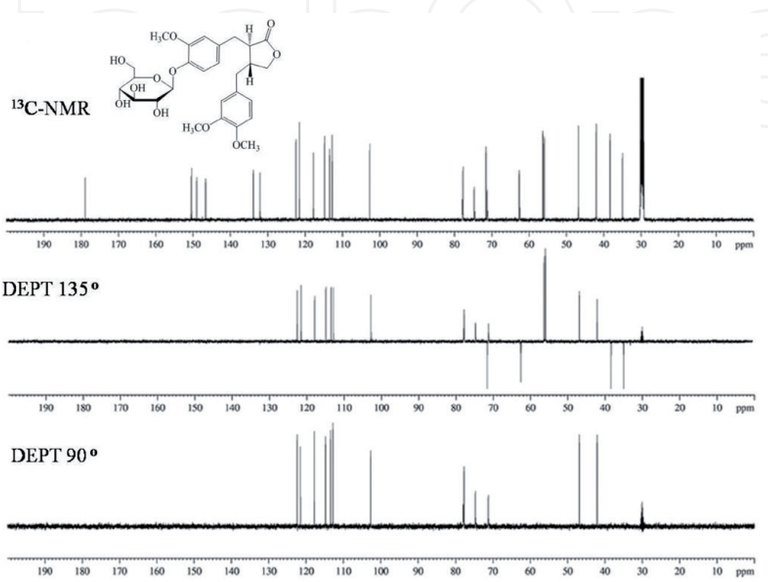

Figure 5.
The DEPT spectrum of Arctiin (CD_3OD).

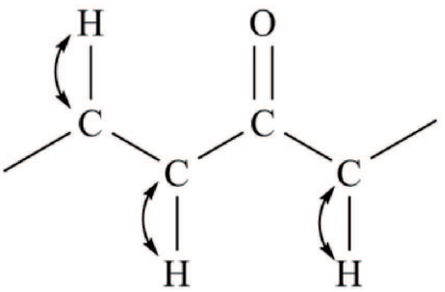

Figure 6.
Schematic diagram of correlations between ^{1}H and ^{13}C in the HSQC or HMQC spectrum.

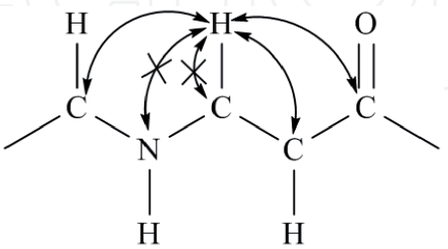

Figure 7.
Schematic diagram of correlations between ^{1}H and ^{13}C in the HMBC spectrum.

In addition, for compounds of aromatic systems, double bond systems, and some particular configuration systems, ^{1}H-^{1}H COSY spectra can show ^{4}J coupling or longer coupling relationships of hydrogen groups. It is very important for the elucidation of an unknown structure.

4.3.4.4.2 HSQC (HMQC) spectrum

^{1}H detected heteronuclear single quantum coherence (HSQC) and ^{1}H detected heteronuclear multiple quantum coherence (HMQC) can display the correlations between ^{1}H and ^{13}C. HSQC possesses higher sensitivity and wider application than HMQC. In the HMQC or HSQC spectrum, the signals occurred at the crosses of chemical shifts generated by corresponding carbons and protons (**Figure 6**).

4.3.4.4.3 HMBC spectrum

HMBC spectrum is short for ^{1}H detected heteronuclear multiple bond correlation, which associates the ^{1}H nucleus with ^{13}C nucleus of long-range coupling. HMBC could detect the long-range coupling of ^{1}H-^{13}C sensitively ($^{n}J_{CH}$, $n \geqq 2$). Moreover, the correlation signal peaks between protons and quaternary carbons that are two or three bonds apart could also be shown in HMBC spectra, as shown in **Figure 7**. From the HBMC spectrum, we can get the connection information of the carbon chain skeletons, the structure information of the quaternary carbons, and the structural information of the coupling systems that are cut off by heteroatoms.

4.3.4.4.4 NOESY spectrum

When two groups of protons are located at rather close spatial distances, irradiation of one group will enhance the signal strength of another, which is known as nuclear Overhauser enhancement (NOE). The NOE spectrum can determine the spatial relative position, stereoscopic configuration, and dominant conformation of some groups in the molecule, which is very important for the study of the stereostructures of organic compounds.

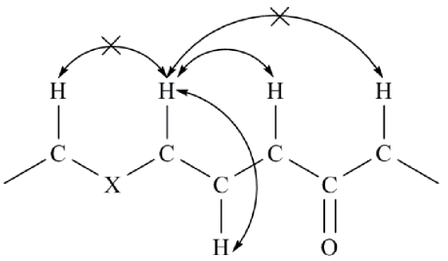

Figure 8.
Schematic diagram of correlations between ^{1}H and ^{13}C in the TOCSY spectrum.

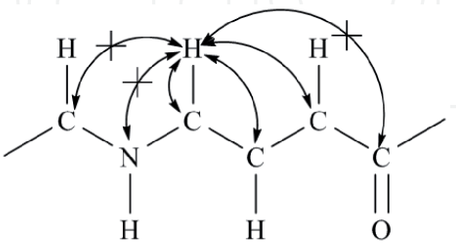

Figure 9.
Schematic diagram of correlations between ^{1}H and ^{13}C in the HSQC-TOCSY spectrum.

2D-NOE (NOESY) spectra could show the NOE correlations of protons. The greatest advantage of NOESY is that all the NOE information between protons of a compound could be shown in one spectrum. However, not all the cross peaks are NOE correlation signals, the residual correlation signals of COSY are often shown in NOESY spectrum as well, which should be paid attention during spectroscopic analysis.

4.3.4.4.5 Total correlation spectroscopy (TOCSY) spectrum

The TOCSY spectrum shows the correlation of the entire spin system, which is different from the ordinary ^{1}H-^{1}H COSY. The relationships between the nuclei that generated the correlation peaks are shown in **Figure 8**. Not only the correlation signals of a proton with protons connected to the adjacent carbons, but also its correlation signals with other protons in a whole spin system could be shown in the TOCSY spectrum, which provides important basis for the connection of structural fragments.

4.3.4.4.6 HSQC-TOCSY spectrum

HSQC-TOCSY is a kind of combined 2D-NMR spectrum. Comprehensive results of HSQC and HMBC are obtained by using a long pulse sequence. The correlation is shown in **Figure 9**. It is very useful for the assignment of carbon and proton signals in complex chemical structures. For example, for saponins with a series of glycosyl groups, the signals generated by glycosyl groups are often overlapped seriously in common NMR spectra, which causes difficulty to assign signals of glycosyls. HSQC-TOCSY spectrum will play an important role in this case. The spectrum includes the information of HSQC, HMBC, and ^{1}H-^{1}H COSY.

4.3.5 Optical rotary dispersion (ORD) and circular dichroism (CD)

Polarimetry is an optical method used widely in the studies of asymmetric structures, which appeared very early. The progress of the sensitive method such as ORD and CD made it possible to study stereostructures of chiral compounds more deeply. Both of them are spectra related to the optical activity of compounds, and

could provide information of absolute configurations, dominant conformations, and reaction mechanisms of chiral compounds, that cannot be replaced by any other spectroscopic methods [28].

4.3.5.1 Optical rotary dispersion (ORD) spectrum

The specific rotation [α] of a chiral compound depends upon the wavelength of the monochromatic light wave. The measurement of specific rotation as a function of wavelength is called optical rotator dispersion (ORD). The common types of ORD curves are as follows.

4.3.5.1.1 Plain curves

The ORD spectrum of an optically active compound with no chromophores is plain without peaks and troughs. An ORD curve of specific rotation increases with decrease of wavelength which is called positive plain curve, while in the case of negative plain curve, negative rotation increases with decrease of wavelength (see **Figure 10**).

4.3.5.1.2 The cotton effect curve

If there is a simple chromophore in the molecule, the ORD curve is very different from plain curve. Near the absorption wavelength region of chromophore, a peak and a trough are exhibited, which is called the Cotton effect, and the spectrum drawn is called the Cotton effect curve. The spectrum with only one peak and one trough is called pure Cotton effect curve, while the spectrum with several peaks and troughs is called complex Cotton effect curve. The Cotton effect is called positive when the trough is observed at a shorter wavelength then peak. Conversely, the Cotton effect is called negative if the trough is observed at a longer wavelength than the peak. Cotton curves of $\triangle^5$-cholestenone are shown in **Figure 11,** which shows A and B possess the same structural formula, while different opposite configurations.

4.3.5.1.3 Complex Cotton effect curve

For compound with two or more different chromophores, its ORD curve may possess multiple peaks and troughs, which is called complex Cotton effect curve. Each ORD curve is the average effect of each chromophore in the molecule, and the contribution of each orientation and conformation of the molecule. Hence the Cotton effect curve is often complex.

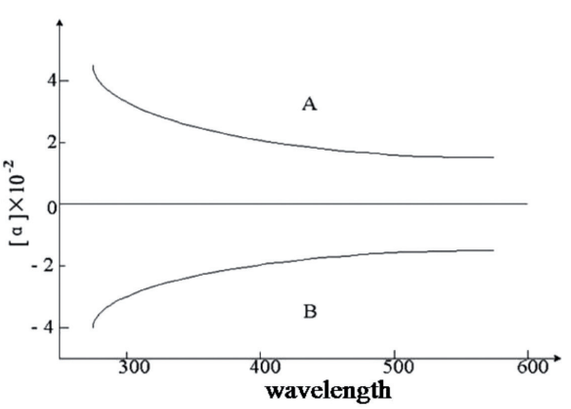

Figure 10.
ORD plain curves (A: Positive plain curve; B: Negative plain curve).

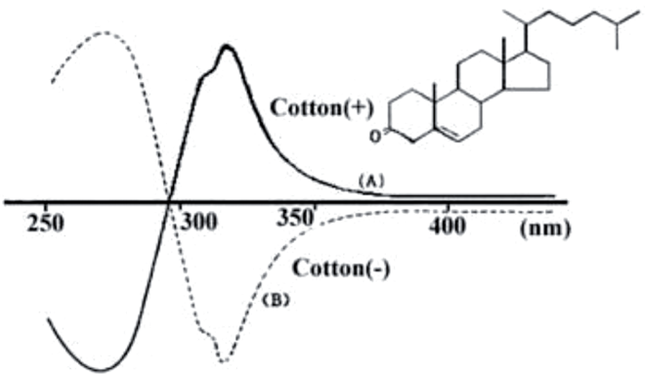

Figure 11.
The Cotton effect curves of △5-cholestenone (A) natural cholesterone (+) cotton; (B) Cholesterone in the opposite absolute configuration (−) cotton.

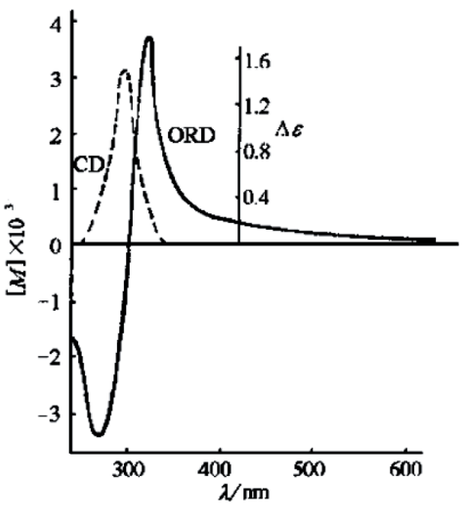

Figure 12.
The ORD and CD spectrum of (+)-camphor.

4.3.5.2 Circular dichroism (CD) spectrum

Optically active compounds have different molar absorption coefficients for left-circularly and right-circularly polarized light that make up plane polarized light, which is called circular dichroism (CD). The difference value between the two molar absorption coefficients ($\Delta\epsilon = \epsilon_L - \epsilon_R$) changes with the wavelength of the incident polarized light. With $\Delta\epsilon$ as the ordinate, the wavelength as the abscissa, the spectrum obtained is called circular dichroism spectrum. Because the absolute value of $\Delta\epsilon$ is very small, it is often replaced by molar ellipticity $[\theta]$. The relationship between $[\theta]$ and $\Delta\epsilon$ is as follows.

$$[\theta] = 3300\Delta\epsilon. \quad (1)$$

Because $\Delta\epsilon$ could be positive or negative, the circular dichroism curve also could be classified as positive and negative. In the CD spectrum showing positive Cotton effect, only a peak appears near the λ_{max} of the chromophore in the molecule. Conversely, a trough appears in the CD spectrum showing negative Cotton effect. Therefore, CD spectra are simpler and easier to analyze than ORD spectra. For example, the ORD and CD spectra of (+)-camphor are shown in **Figure 12**. CD is more widely used than ORD in the study of chiral compounds.

4.3.6 Single crystal X-ray diffraction method

Single crystal X-ray diffraction could be applied independently to analyze the structures, components, contents, configurations, conformations, solvents, and crystal forms of samples. It is widely used in the stereostructural study of natural compounds, synthetic compounds, peptides, proteins, etc. Therefore, X-ray diffraction analysis is a necessary physical method in the field of structure and function research of modern natural drugs.

Single crystal X-ray diffraction is a kind of quantitative analysis technology, which can provide three-dimensional structural information of molecules, including atomic coordinates, bond length, bond angles, dihedral angles, hydrogen bonds, salt bonds, coordinate bonds, and so on. In addition, it is also a reliable method to determine the absolute configuration of chiral drug molecules and the epimers in the stereochemical structures. For example, see [29].

5. Conclusions and future directions

In recent years, study on phytochemicals from plants becomes more and more popular due to their demonstrated health benefits. A number of plants having high contents of phytochemicals (particularly phenolic acids and flavonoids) with associated antioxidant activities have been increasingly utilized. Complementary research is also needed to enhance the potential functionalities of the phytochemicals in future, where such plants have shown to contain numerous phytochemicals that may be beneficial to human health. The compiled results indicated that many of their bioactive compounds remain to be fully isolated, identified, and characterized (alkaloids, diterpenoids, and so on).

Therefore, phytochemicals can be considered as the source of natural medicines. The compounds of plants are bioaccessible and bioavailable in humans with some demonstrated health benefits, including antioxidant, anti-inflammatory, anti-cancer, anti-microbial, hypoglycemic action, etc. Additional well-designed human intervention studies and clinical trials are needed to validate the health benefits of phytochemicals.

Author details

Weisheng Feng*, Meng Li, Zhiyou Hao and Jingke Zhang
School of Pharmacy, Henan University of Chinese Medicine, Zhengzhou, China

*Address all correspondence to: fwsh@hactcm.edu.cn

References

[1] Brusotti G, Cesari I, Dentamaro A, Caccialanza G, Massolini G. Isolation and characterization of bioactive compounds from plant resources: The role of analysis in the ethnopharmacological approach. Journal of Pharmaceutical and Biomedical Analysis. 2014;**87**:218-228. DOI: 10.1016/j.jpba.2013.03.007

[2] Alberti Á, Riethmüller E, Béni S. Characterization of diarylheptanoids: An emerging class of bioactive natural products. Journal of Pharmaceutical and Biomedical Analysis. 2018;**147**:13-34. DOI: 10.1016/j.jpba.2017.08.051

[3] Hosler DM, Mikita MA. Ethnobotany: The chemist's source for the identification of useful natural products. Journal of Chemical Education. 1987;**64**:328-332

[4] Silverstein RM, Bassler GC. Spectrometric Identification of Organic Compounds. ACS Publications; 1962

[5] Luo YM. Technology and Method of Extraction and Separation of Chemical Constituents of Traditional Chinese Medicine. Shanghai, China: Shanghai Scientific & Technical Publishers; 2016

[6] Gray AI, Igoli JO, Edrada-Ebel R. Natural products isolation in modern drug discovery programs. Natural Products Isolation. 2012;**864**:515-534. DOI: 10.1007/978-1-61779-624-1_20

[7] Tyśkiewicz K, Konkol M, Rój E. The application of supercritical fluid extraction in phenolic compounds isolation from natural plant materials. Molecules. 2018;**23**:2625. DOI: 10.3390/molecules23102625

[8] Rostagno MA, Palma M, Barroso CG. Ultrasound-assisted extraction of soy isoflavones. Journal of Chromatography A. 2003;**1012**:119-128. DOI: 10.1016/S0021-9673(03)01184-1

[9] Nayak B, Dahmoune F, Moussi K, Remini H, Dairi S, Aoun O, et al. Comparison of microwave, ultrasound and accelerated-assisted solvent extraction for recovery of polyphenols from *Citrus sinensis* peels. Food Chemistry. 2015;**187**:507-516. DOI: 10.1016/j.foodchem.2015.04.081

[10] Pan XJ, Niu GG, Liu HZ. Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves. Chemical Engineering and Processing. 2003;**42**:129-133. DOI: 10.1016/S0255-2701(02)00037-5

[11] Tang ZH, Guo SY, Rao LQ, Qin JP, Xu XN, Liang YZ. Optimization of the technology of extracting water soluble polysaccharides from *Morus alba* L. leaves. African Journal of Biotechnology. 2011;**10**(59):12684-12690. DOI: 10.5897/AJB10.2203

[12] Koh GY, Chou G, Liu ZP. Purification of a water extract of Chinese sweet tea plant (*Rubus suavissimus* S. lee) by alcohol precipitation. Journal of Agricultural and Food Chemistry. 2009;**57**:5000-5006. DOI: 10.1021/jf900269r

[13] Azmir J, Zaidul ISM, Rahman MM, Sharif KM, Mohamed A, Sahena F, et al. Techniques for extraction of bioactive compounds from plant materials: A review. Journal of Food Engineering. 2013;**117**:426-436. DOI: 10.1016/j.jfoodeng.2013.01.014

[14] Tahara S, Yamamoto S, Yamajima Y, Miyakawa H, Uematsu Y, Monma K. A rapid dialysis method for analysis of artificial sweeteners in foods (2nd report). Shokuhin Eiseigaku Zasshi. 2017;**58**(3):124-131. DOI: 10.3358/shokueishi.58.124

[15] Hanif MA, Nawaz H, Naz S, Mukhtar R, Rashid N, Bhatti IA, et al. Raman spectroscopy for the characterization of different fractions of hemp essential oil extracted at 130°C using steam distillation method. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2017;**182**:168-174. DOI: 10.1016/j.saa.2017.03.072

[16] Escribà-Gelonch M, Hessel V, Maier MC, Noël T, Neira d'Angelo MF, Gruber-Woelfler H. Continuous-flow in-line solvent-swap crystallization of vitamin D_3. Organic Process Research and Development. 2018;**22**:178-189. DOI: 10.1021/acs.oprd.7b00351

[17] Poole CF. Chromatographic test methods for characterizing alkylsiloxane-bonded silica columns for reversed-phase liquid chromatography. Journal of Chromatography B. 2018;**1092**:207-219. DOI: 10.1016/j.jchromb.2018.06.011

[18] Porath J. From gel filtration to adsorptive size exclusion. Journal of Protein Chemistry. 1997;**16**:463-468

[19] Gerberding SJ, Byers CH. Preparative ion-exchange chromatography of proteins from dairy whey. Journal of Chromatography. A. 1998;**808**:141-151

[20] Ji S, Wang S, Xu H, Su Z, Tang D, Qiao X, et al. The application of on-line two-dimensional liquid chromatography (2DLC) in the chemical analysis of herbal medicines. Journal of Pharmaceutical and Biomedical Analysis. 2018;**160**:301-331. DOI: 10.1016/j.jpba.2018.08.014

[21] Cardoso CAL, Wilegas W. Droplet counter-current chromatography of indole alkaloids from *Tabernaemontana hilariana*. Phytochemical Analasis. 1999;**10**:60-63. DOI: 10.1002/(SICI)1099-1565(199903/04)10:2<60:AID-PCA446>3.0.CO;2-Y

[22] Gu M, Ouyang F, Su ZG. Comparison of high-speed counter-current chromatography and high-performance liquid chromatography on fingerprinting of Chinese traditional medicine. Journal of Chromatography. A. 2004;**1022**:139-144. DOI: 10.1016/j.chroma.2003.09.038

[23] Horie H, Kohata K. Analysis of tea components by high-performance liquid chromatography and high-performance capillary electrophoresis. Journal of Chromatography. A. 2000;**881**(1-2):425-438. DOI: 10.1016/S0021-9673(99)01345-X

[24] Cao H, Liu XJ, Ulrihc NP, Senguptad PK, Xiao JB. Plasma protein binding of dietary polyphenols to human serum albumin: A high performance affinity chromatography approach. Food Chemistry. 2019;**270**:257-263. DOI: 10.1016/j.foodchem.2018.07.111

[25] Kumar K, Siva B, Rama Rao N, Suresh Babu K. Rapid identification of limonoids from *Cipadessa baccifera* and *Xylocarpus granatum* using ESI-Q-ToF-MS/MS and their structure-fragmentation study. Journal of Pharmaceutical and Biomedical Analysis. 2018;**152**:224-233. DOI: 10.1016/j.jpba

[26] Monakhova YB, Kuballa T, Lachenmeier DW. Chemometric methods in NMR spectroscopic analysis of food products. Journal of Analytical Chemistry. 2013;**68**:755-766

[27] Bakiri A, Hubert J, Reynaud R, Lanthony S, Harakat D, Renault JH, et al. Computer-aided ^{13}C NMR chemical profiling of crude natural extracts without fractionation. Journal of Natural Products. 2017;**80**:1387-1396. DOI: 10.1021/acs.jnatprod.6b01063

[28] Wang J, Yang XD. Determination of absolute configuration of chiral compounds based on chiroptical

spectroscopic methods: From instrument characterization to computational chemistry. University Chemistry. 2016;**31**:37-44. DOI: 10.3866/PKU.DXHX201603022

[29] Zhang D, Guo J, Zhang M, Liu X, Ba M, Tao X, et al. Oxazole-containing diterpenoids from cell cultures of *Salvia miltiorrhiza* and their anti-HIV-1 activities. Journal of Natural Products. 2017;**80**:3241-3246. DOI: 10.1021/acs.jnatprod.7b00659