Published in final edited form as:

Anal Lett. 2014 ; 47(11): 1835–1851. doi:10.1080/00032719.2014.888727.

RECENT ADVANCES IN ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE ANALYSIS OF TRADITIONAL CHINESE MEDICINE

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Abstract

Traditional Chinese medicine has been widely used for the prevention and treatment of various diseases for thousands of years in China. Ultra-high performance liquid chromatography (UHPLC) is a relatively new technique offering new possibilities. This paper reviews recent developments in UHPLC in the separation and identification, fingerprinting, quantification, and metabolism of traditional Chinese medicine. Recently, the combination of UHPLC with MS has improved the efficiency of the analysis of these materials.

Keywords

UHPLC; Mass Spectrometry; TCM; ultra-high performance liquid chromatography

INTRODUCTION

In 2004, the first commercially available ultra-high performance liquid chromatography (UHPLC) instrument, the Acquity System, became commercially available for separation of small organic molecules, proteins, and peptides (Swartz 2004a, 2004b). UPLC (ultra-high performance liquid chromatography) is a trade name of the Waters Corporation for its system. Using a column packed with 1.7 μ m particles, UPLC leads to higher separation efficiency for the analysis. At its introduction Waters proclaimed UPLC means “speed, resolution, and sensitivity” (Waters Corporation 2004). UHPLC instruments operate at pressures up to 15,000 psi with faster data collection in comparison to conventional HPLC. Soon after, other liquid chromatography manufacturers developed UHPLC systems. Compared with conventional high-performance liquid chromatography (HPLC), UHPLC gives improved resolution, faster analysis speed, and better sensitivity. It is especially useful for the analysis of traditional Chinese medicine since these materials are complex matrices and the resolution is often insufficient using conventional HPLC. For these reasons, UHPLC currently is widely used for the analysis of traditional Chinese medicines (TCMs).

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It is well known that TCMs play an important role in the prevention and treatment of diseases in China (Efferth 2011). TCMs, usually a cocktail of herbal ingredients, have many active constituents and their respective concentrations can be influenced by breeds, origins, growing conditions, and processing techniques. Their effectiveness is commonly considered to involve synergistic effects of all components. Quality control of TCMs is a challenge due to their complexity. UHPLC provides high quality data to identify active compounds in TCM.

UHPLC-Diode Array Detector (UHPLC-DAD) Applications

In comparing two official Chinese pharmacopoeia species of *Ganoderma*, determination of triterpenes using UHPLC with a diode array detector (DAD) provided identification and quantitation (Da et al. 2012). A method using UHPLC with a DAD detector (UHPLC-DAD) separated about 30 phenolic compounds in *Matricaria chamomilla* and its crude extracts with a run time about two times faster than a conventional HPLC method (Jing et al. 2013). Moreover, UHPLC requires smaller amounts of solvents than the HPLC approaches (Haghi et al. 2014).

UHPLC-MS Applications

Although there are UHPLC-DAD applications as mentioned in the previous section, UHPLC excels when coupled with mass spectrometry (MS) (Liang et al. 2013; Xu et al. 2012; Wang et al. 2012; Lou et al. 2010). Mass spectrometry is widely used for compound identification and is the most selective detector for compounds from natural products. Mass spectrometers can be classified as HRAM (high resolution accurate mass)-MS and conventional MS, based on resolution and mass accuracy of their mass analyzers. UHPLC diode array detector (DAD) –MS is very useful for identifying target compounds in TCMs by comparing retention times, ultraviolet absorbance values, molecular ions, and fragmentation data, with or without reference standards. UHPLC coupled with high resolution accurate mass spectrometry (UHPLC-HRAM-MS) reduces analysis time and identifies compounds.

Ninety-two compounds including ginsenosides, lignans, steroidal saponins and homoiso-flavanones were identified by UHPLC-HRAM-MS in Sheng-mai San. This method shows high sensitivity and resolution (Wu et al. 2011). The application of the UHPLC-ESI-MS/MS method allowed the determination of hydroxy fatty acids and phenylethanoids with satisfactory precision (Yang et al. 2013; Gryszczy ska et al. 2012). The HRAM-MS analytical platform has been shown to be fast, sensitive, and reliable for the determination of small molecules in complex matrices (Malik et al. 2010).

UHPLC-Quadrupole Time-of-flight (QTOF) MS—The performance of TOF analyzers has greatly improved in recent years in terms of resolution and mass accuracy (Domon and Aebersold 2006). The combined quadrupole (Q) Q-TOF instrument exhibits high resolution and mass accuracy in MS and MS/MS modes. In MS mode, the quadrupole acts as an ion guide to the TOF analyzer. In MS/MS mode, the precursor ions are selected in the first quadrupole and undergo fragmentation through collision induced dissociation, with product ions analyzed in the TOF device. The combination of UHPLC and Q-TOF technique has

accelerated the screening of compounds, while giving excellent separation and good structural characterization abilities which make it suitable to analyze traditional Chinese medicines.

UHPLC-QTOF-MS was used to identify and evaluate a series of components from TCMs, such as coumarin isomers (Lv et al. 2012), phloroglucinol derivatives, diterpenes (Wang et al. 2013), biogenic amines (Jia et al. 2012), quinovic acid glycosides (Pavei et al. 2012), and alkaloids (Jiang et al. 2012). Gahlaut et al. used UHPLC-QTOFMS to distinguish and validate various *Saraca asoca* extracts and its herbal formulation 'Ashok arista' (Gahlaut et al. 2013). Forty components belonging to monoterpene glucosides, galloylglucose, and phenolic compounds of *Radix paeoniae* were identified by UHPLC-PDA-QTOF-MS (Li et al. 2009).

UHPLC-QTOF-MS/MS has been also applied to separate and characterize steroidal saponins in crude extracts from *Dioscorea zingiberensis*. A total of thirty-one saponins with five aglycone skeletons, including fourteen new trace saponins, were identified or tentatively elucidated based on their retention times, the mass spectrometric fragmentation patterns, and MS and MS/MS data (Zhu et al. 2010). Similarly, Ji et al. developed a rapid method for xanthenes and steroidal saponins of *Rhizoma Anemarrhenae* (Ji et al. 2012). It was also used for the rapid identification of polymethoxylated flavones in *Fructus aurantii* (Zhou et al. 2009) and revealed chemical transformation of sulfur-fumigated herbs or the decoction of herbs such as Du–Shen– Tang (Li et al. 2012; Song et al. 2010). In a recent study by Cádiz-Gurrea et al., UHPLCQTOF-MS was used to characterize phenolic compounds in the leaves and flowers of *Eryngium bourgatii* (Cádiz-Gurrea et al. 2013). It was also used to rapidly screen and identify steroidal saponins from the seeds of *Trigonella foenum-graecum* and the extract of *Paris Polyphylla* var. *Yunnanensis* (Kang et al. 2013a, 2012b).

UHPLC-QTOF was also used for the analysis of Chinese medicine formulae. Eighty-one major constituents such as organic acids, amino acids, oligosaccharides, and alkaloids were separated and identified in Shenqi-Fuzheng injection (Liu et al. 2013). Twenty-two compounds of Suan-zao-ren decoction were tentatively identified using UHPLC-ESI-QTOF-MS (Yang et al. 2011). Furthermore, UHPLC-QTOF MS was used to elucidate chemical differences between traditional and dispensing granule decoctions of Tao-Hong-Si-Wu decoction (Shang et al. 2012). In the analysis of aconitum alkaloids in Yin-chen-si-ni decoction, 62 ions were assigned to aconitum alkaloids and identified tentatively by comparing the accurate mass and fragments information with authentic standards or literature reports (Yan et al. 2010). It is also a useful method for finding global chemical differences between traditional and dispensing granule decoctions, and the most changed components during the decoction of San–Huang–Xie–Xin–Tang were identified (Li et al. 2010).

UHPLC-Linear Ion Trap (LTQ)-Orbitrap-MSⁿ—This hybrid configuration provides enhanced selectivity and multiple scan types. Furthermore, no internal calibration is needed to obtain high mass accuracy resulting in a simplified experimental protocol. Based on the HRAM mass information, MS/MS fragmentation behavior, and the literature, forty-three compounds, including new types of saponin aglycones, and malonyl-substituted and acetyl-

substituted saponins, were identified in *Panax notoginseng* (Xu et al. 2012). A total of forty-two diester-diterpenoid alkaloids were separated and identified in the roots of *Aconitum carmichaeli* by UHPLC/MSⁿ. This method has been demonstrated to be an effective tool for the analysis of TCMs (Zhang et al. 2012). Thirty-three compounds, including catechin derivatives, flavanones, phenolic acid derivatives, and phenylpropanoid glycosides were either identified or tentatively characterized in the rhizome of *Smilacis glabrae* (Li et al. 2012). The same method was used for the identification of bioactive constituents in *Sarcandra glabra* and its preparations. Fifty compounds, including organic acids, caffeoyl derivatives, flavonoids, coumarins and terpenoids were identified (Li et al. 2011).

Quantitative Analyses of Targeted Components of TCMs—It is well known that the synergistic actions of TCMs are caused by their multiple constituents. Multi-component quantitative analysis may provide systematic information about TCMs. Many methods using UHPLC with different detection technologies have been used for quantitative analysis of TCMs (Liu et al. 2012; Liang et al. 2010). UHPLC offers reduced analysis time and allows the determination of more compounds compared with conventional HPLC (Kuang et al. 2011). UHPLC-DAD was applied to investigate panaxyrins A and B from roots of *Panax ginseng* (Araliaceae) (Lee et al. 2010). Components quantified by this method included flavonoids, rhodiosin, curcuminoids, flavonoid glycosides, and phenylethanoid glycosides (Cao et al. 2012; Zhu et al. 2013; Cheng et al. 2010; Li et al. 2012; Zhao et al. 2013). This method was developed for the determination of narirutin, naringin, hesperidin, neohesperidin, nobiletin, and tangeretin in *Fructus aurantii*, which provided high chromatographic resolution and rapid analysis (less than 8 min). The samples were also analyzed by UPLC/ESI-Q-TOF-MS to confirm the identification results (Cao et al. 2012).

Rhodiola rosea has been used as an adaptogen in Chinese traditional medicine for centuries. An efficient ionic liquid-based online ultrasonic assisted extraction (UAE) and solid-phase trapping (SPT) method was developed for selective extraction and UPLC quantification of rhodiosin and rhodionin from *R. rosea*. Cheng et al. developed an UPLC-DAD method, using a C₁₈ (2.1mm × 100mm, 1.7μm) column for rapid quantitation of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in *Curcuma longa* with an analysis time of 2 min (Cheng et al. 2010). A comprehensive UPLC-DAD method was established to determine the presence of five characteristic phenylethanoid glycosides in the original plants and various commercial products of small-leaved Kudingcha (*Ligustrum robustum*) and to distinguish this species from similar species, such as the *L. henryi*, *L. lucidum*, and *L. pricei* (Li et al. 2012).

Yang et al. combined nanomagnetic powder three-phase hollow fiber-based liquid-phase microextraction (HF-LPME) with UHPLC-MS for the analysis of nine flavonoids in *Polygonum hydropiper*, and the results indicate that this method has low detection limits, good reproducibility, and a wide linear range (Yang et al. 2011). A total of twenty-four bioactive components including flavonoid glycosides, terpene lactones, biflavones, proanthocyanidins, and ginkgolic acids were quantified in *Ginkgo biloba* leaves based on a UHPLC-MS/MS method. (Yao et al. 2013). Zearalenone, saponins, and gingerols were determined efficiently using UHPLC-MS (Han et al. 2011; Cheng et al. 2013). Ping Cui et

al. applied UHPLC-MS to determine mesaconitine, aconitine, hypaconitine, benzoylmesaconitine, benzoylhypaconitine, and benzoylaconitine (Cui et al. 2012). A method using UHPLC-QTOF-MS was developed for the determination of ginsenosides Rb₁, Rb₃, Rd, and F₂ in *Gynostemma pentaphyllum* in order to distinguish the sweet and bitter taste variants of a Chinese medicinal tea (Lu et al. 2013).

The determination of many complex components of traditional Chinese preparations like Si-Jun-Zi-Tang, Samhwangsasim-tang, Xue Fu Zhu Yu and Dan-Lou tablets have also been achieved (Zhang et al. 2011; Poudel et al. 2013; Zhang et al. 2012; Dong et al. 2013). Liang et al. combined a UHPLC with a tunable UV detector (UHPLCTUV) and a UHPLC-Q-TOF method for identification and quantification of the main constituents in Niu-Huang-Jie-Du tablets. Ten compounds responsible for the therapeutic effects were determined (Liang et al. 2010). In the analysis for 'Ge-Gen-Qin-Lian' tablets and 'Jiang-Zhi' granules, UHPLC-MS rapidly determined some components (An et al. 2009; Lu et al. 2010).

Fingerprint Analyses of TCMs by UHPLC

Because TCMs are composed of multiple constituents, quality control based on the quantitative analysis of a few marker compounds does not reflect their overall quality. Consequently, chromatographic fingerprinting is recommended by the Chinese Pharmacopeia (Steinmann et al. 2011; Guan et al. 2011). Fingerprint analysis has also been accepted by the WHO for the assessment of herbal medicines (Jiang et al. 2010). In 2004, "fingerprints" of liquid injections of TCMs were mandated by the Chinese State Food and Drug Administration (SFDA) for quality control (Liang et al. 2004).

UHPLC-MS is a useful approach for rapid pharmaceutical analysis for the differentiation of origin, the determination of authenticity, and the overall quality assessment of herbal medicines. Typical examples for single herb fingerprinting include *Physalis alkekengi* (Zheng et al. 2012), *Radix aconiti* (Zhu et al. 2013), *Ilex latifolia* (Fan et al. 2013), *Magnoliae officinalis* (Wang et al. 2010) and *Rhizoma Coptidis chinensis* (Kong et al. 2009). Combined with UHPLC and DAD, an established approach to the structural identification of physalins by ESI-MS/MS was applied to the analysis of active extracts of *Physalis alkekengi* from different locations (Zheng et al. 2012). Zhu et al. established a UHPLC-ESI/MSⁿ fingerprinting approach in combination with chemometric analysis for the quality control of processed *Radix aconiti*. The results showed that the well processed, unqualified processed, and the raw *Radix aconiti* clustered corresponding to their constituents by a flexible and reliable method (Zhu et al. 2013).

UHPLC-MS fingerprinting methods are successfully applied for quality assessment of TCMs and to identify major components. Examples include the fingerprint analysis of traditional Chinese formulae like Si-wu decoction (Su et al. 2013), Si-Jun-Zi-Tang (Wang et al. 2013), and Yuan-hu Zhi-tong Tablet (Xu et al. 2013). Eighty-four components were identified or characterized, including ten organic acids, thirty glycosides (monoterpene or iridoid or phenylpropanoids glycosides), fourteen lactones, eighteen flavonoids, and eleven alkaloids in Siwu decoction (Su et al. 2013). A UPLC-QTOF-MS-MS method was used to determine sixty-six major compounds in Si-Jun-Zi-Tang; fifty-eight were tentatively identified. Most major constituents including ginsenosides, licoflavones, and glycyrrhizin in

n-butanol extract were studied from *Radix ginseng* and *Glycyrrhiza uralensis* (Wang et al. 2013).

Metabolomics by UHPLC in TCMs

Metabolomics, which is the comprehensive assessment of endogenous metabolites of a biological system, is a promising tool for biomarker discovery. The metabolomic approach has done much to further the explore TCMs, and plays an important role for high-throughput elucidation of metabolic phenotypes of crude and processed preparations. Metabolic profiling changes analyzed by UHPLC–MS–MS can be very helpful in understanding TCMs. Chao Ma reported an UHPLC–MS metabolomic approach to characterize the profile of plasma and kidney extract from rats treated with *Morning Glory seeds*. The results indicate that lysophosphatidylcholine formation and the sphingolipid cycle were accelerated, while the phenylalanine level in serum decreased (Ma et al. 2010). A metabolomic method was developed to establish the profiles of rat plasma to investigate the anti-osteoporosis effect of *Rhizoma drynariae* and its mechanism. Six potential metabolite biomarkers were identified, including lysophosphatidylcholines, tryptophane, and phenylalanine (Liu et al. 2012). Lu studied the urine metabolite profiling of ‘Kidney-Yang Deficiency syndrome’ induced by hydrocortisone and the positive effect of *Rhizoma drynariae* in rats by UHPLC–MS (Lu et al. 2011).

There are several UHPLC–Q–TOF/MS reports of metabolomic studies investigating the action mechanism of traditional Chinese medicine. UHPLC–Q–TOF with automated data analysis was applied for rapid analysis of hyperoside metabolites in rat tissue after intravenous administration to understand the mechanism of this compound (Guo et al. 2011). UHPLC–QTOF–MS–based screening has also been used for determining the constituents and their metabolites in rat plasma and urine after oral administration of the *Glechoma longituba* extract (Ni et al. 2010) and Ping-gan prescription (Jiang et al. 2012). Ultra-performance liquid-chromatography/electrospray ionization synapt high-definition mass spectrometry combined with pattern recognition approaches including principal component analysis (PCA) were integrated to approximate the comprehensive metabolic signature and discover differentiating metabolites. These practices are promising for profiling TCMs and using the metabolomic platform (Yang et al. 2012). UHPLC–HRAM–MS combined with a principal component analysis (PCA) was used for exploring the excretion pattern of low molecular mass metabolites (Wang et al. 2010), to study the metabolomic characteristics and the micromolecular marking compound of urine, and to study the efficacy and mechanism of complex TCM prescriptions with a rapid and noninvasive urinary metabolomics approach (Su et al. 2011; Sun et al. 2010).

Aconite root, an herbal medicine in TCM, has a very narrow therapeutic range. An approach based on UHPLC–HRAM–MS with a metabolomic approach was employed to demonstrate the plasma characteristics, speculate on alternative pathways, and compare differences between the crude and processed products. The study provides information to further understand the pharmacological activity and potential toxicity of processed *Aconite* roots (Wang et al. 2012). Pyrrolizidine alkaloids show significant hepatotoxicity as they bind to DNA or proteins after activation in the liver. A metabolomic study using UHPLC–MS to

obtain the profiles of *Senecio scandens* and *S. vulgaris* was reported. This study provided a better understanding of the toxicities of two *Senecio* herbs containing pyrrolizidine alkaloids (Xiong et al. 2011)

Pharmacokinetic Applications by UHPLC in TCMs

The traditional approach of determining metabolites from biological samples involves isolation by column chromatography or preparative HPLC unless reference standards are available. It is a very expensive, laborious, and time-consuming process. UHPLC-HRAM-MS can provide information on the structure of the metabolites much faster, especially when a database is available. Numerous reports have shown the use of UHPLC to explore the efficacy and mechanism of TCMs.

UHPLC-MS pharmacokinetic studies of TCMs have been performed by many groups (Yang et al. 2012; Li et al. 2012; Qiu et al. 2011; Yin et al. 2012; Gan et al. 2012; Xiong et al. 2010; Wang, et al. 2011; Sun et al. 2012). A pharmaco-metabolomics study to explore the therapeutic basis and to clarify the mechanism of traditional Chinese medicine by UHPLC-MS has also been reported (Sun et al. 2013; Zhang et al. 2011).

Pharmacokinetic studies on active constituents in TCMs are used to explain and predict the efficacy and toxicity. A rapid, sensitive and selective UHPLC-MS-MS method was developed for determination of the bioactive components and their metabolites in rat plasma after the administration of Shaofu Zhuyu decoction. Nine metabolites were identified in rat plasma based on the mass fragmentation and literature reports (Su et al. 2010). Guo et al. investigated the anticonvulsant and antidepressant-like effects of *Abelmoschus manihot* ethanol extract as well as its potential active components *in vivo*. Eight flavonoids were identified in rat brain after its administration by UHPLCMS (Guo et al. 2011).

UHPLC-Q-TOF-MS was used to determine constituents in rat biological fluids after oral administration of Fufang Danshen tablets. *In vivo*, fourteen components and eight metabolites of FDT were observed in plasma, and twelve components were detected in urine (Lv et al. 2010). Zhong et al. used UHPLC-Q-TOF-MS to determine the constituents in rat serum after oral administration of Fufang Zhenzhu Tiaozhi capsules (Zhong et al. 2012). Another UHPLC-HRMS method was developed to analyze and identify the bioactive components and their metabolites in rat plasma following oral administration of *Radix stemonae* extract. The method could be applied to other TCMs (Dong et al. 2012). The structure and metabolite profile of eleutheroside B were elucidated by a UHPLC-Q-TOF MS method (Lu et al. 2012).

UHPLC-MS is a selective and sensitive method that can also be used effectively to quantify bioactive components *in vivo*. Many quantitative methods, including validated methods, were successfully applied to investigate the pharmacokinetics of the bioactive compounds in rats. Puerarin, daidzein, baicalin, wogonoside, and liquiritin of Ge-gen-Qin-lian decoction were determined in rat plasma using UHPLC-MS (Wang et al. 2009). A UHPLC-MS/MS method for koumine determination in rat plasma after a single intravenous dose of 20 mg/kg was validated (Chen et al. 2013). The quantification of ephedrine in *Herba ephedrae* and Wu-Tou-Tang decoctions in rat plasma were also studied using UHPLC-MS (Zheng et al.

2012). Quantitation of ligustilide, dehydrocostuslactone, α -cyperone (Liu et al. 2013), psoralen, isopsoralen (Gu et al. 2009), pimpinellin, isopimpinellin, phellopterin (Liu et al. 2012), paeoniflorin, naringin, naringenin, glycyrrhetic acid (Wen et al. 2012), and three physalins (Zheng et al. 2012) in rats by UHPLC-MS/MS have also been reported.

Tao et al. developed a UHPLC-MS-MS method which was successfully applied to a pharmacokinetic study of eight triterpenoid saponins including ursaponin C, ursaponin F, 22 β -acetoxyl-glycyrrhizin, 24-hydroxy-licorice-saponin E2, licorice-saponin G2, licorice-saponin E2, glycyrrhizin, and licorice-saponin J2 in dog plasma after oral administration of total saponin of licorice (Tao et al. 2013). Wang et al. used an UHPLC-TOF-MS method to determine fifteen ginsenosides and metabolites and their bioavailability in humans. Compared to conventional HPLC method, the UHPLC method has significant advantages in high-speed separation and rapid resolution. Ginsenosides Rb1, Rd, Rg2, and compound K were detected in the plasma samples and enteric microbiota are responsible for metabolism of parent ginsenosides to compound K (C.Z. Wang et al. 2011).

Microdialysis allows the determination of drug concentrations in blood and tissues. This technique may be applied to pharmacokinetic studies without depriving the subject of biological fluids and disturbing physiological functions. Microdialysis was combined with UHPLC-MS to determine ligustilide in rat brains. This method uses fewer animals and demonstrated use with UHPLC-MS for pharmacokinetic studies (Guo et al. 2011). Xue et al. combined microdialysis with UHPLC-Q-TOF-MS to identify components in blood and kidney dialysis after oral administration of *Abelmoschus manihot* extract. This method was simple and reliable, and could be adopted to rapidly screen and identify potential active components contributing to pharmacological effects of TCMs (C.F. Xue et al. 2011).

Preliminary screening, systematic screening and processing technology investigation also can be accomplished by UHPLC. For instance, Kong et al. proposed a novel “target constituent knock-out” strategy coupled for preliminary screening of antibacterial constituents in *Calculus bovis* (Kong et al. 2011). Moreover, systematic screening of tertiary and quaternary alkaloids from *Corydalis yanhusuo* and processing technology investigation of Loquat leaf (*Eriobotrya japonica*) were performed based on UHPLC-QTOF-MS (Zhang et al. 2009; Wu et al. 2013).

CONCLUSIONS

Traditional Chinese medicines have been used for centuries for the prevention and treatment of human disease. Over the past decades, traditional herbal medicines have been an important resource for screening lead compounds. HPLC is a predominant technology used in laboratories worldwide, and made a significant contribution to the modernization of Chinese traditional medicines during the past 40 years. UHPLC is a relatively new technique providing decreased analysis time and solvent consumption. UHPLC retains the practicality and principles of HPLC while providing improvement in performance. It is evident that the use of UHPLC is advantageous in terms of speed for analyses requiring up to 80,000 theoretical plates. This opens the options for reducing the analysis time of current HPLC procedures (Villiers et al. 2006). UHPLC coupled with MS provides accurate mass

measurement, high resolution, and selectivity, and is an important tool for the determination of constituents in TCMs.

Acknowledgments

This research is supported by the Agricultural Research Service of the U.S. Department of Agriculture and an Interagency Agreement with the Office of Dietary Supplements of the National Institutes of Health, the National Natural Science Foundation of China (NSFC) (No.31370376 and No.81160507), and the Project of University's Young Teachers' Development of Jiangxi Province, China (2012-132).

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