



Quality markers as a tool for evaluation of medicinal mushroom, *Cordyceps s.l. (sensu lato)* species during bioprocessing and quality control

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Abstract

Cordyceps militaris and *Ophiocordyceps sinensis* having multiple pharmacological properties are used as expensive traditional medicines worldwide in health and food sector. The bio-efficacy, quality and safety are three significant keys for the assessment of any health and food products. Keeping in mind the plethora of medicinal properties of natural *C. militaris* and *O. sinensis*, many mycelial isolates have been obtained from natural sources and bio-processed using solid state fermentation technology due to its scarcity in nature; which are usually traded as health and food products around the world. The occurrence of counterfeits alongside other substitutes of *C. militaris* and *O. sinensis* with similar pharmacological efficacy has added to the confusion in the market about the authenticity of the products. Consequently, quality control and quality assurance of *C. militaris* and *O. sinensis* and their bio-products have become vital for ensuring its safety and bio-efficacy. Following standard protocol for quality control is critical to confirm the authenticity and medicinal value of *C. militaris* and *O. sinensis*. Quality markers chosen with regard to its bio-efficacy and safety should ideally be considered as the most rational markers. Now days, several markers have been suggested for quality control of cordyceps such as nucleosides, mannitol, ergosterol, proteins and polysaccharides. However, such markers need to be studied thoroughly in terms of their commercial aspects and scientific efforts are needed to assign active ingredients' contribution to the pharmacological efficacy. Herein, various markers and analytical methods to identify them are reviewed and discussed, which would add to the existing information on cultivation of these mushroom with special reference to the advancements and challenges relevant to accelerating quality control measures in the bioprocess.

Key words – Analytical techniques – cordyceps – HPLC – nucleosides – polysaccharides – quality marker – quality control and quality assurance

Introduction

Cordyceps s.l. (sensu lato) species, also known as “soft gold” or “cordyceps”, is a much-valued traditional Chinese medicine (TCM) with multiple health benefits and pharmacological properties which has come up as a big industry worldwide and China, in particular, in recent years (Yue et al. 2013). There are 750 identified species of cordyceps worldwide today, out of which those which are predominantly used for their medicinal properties, health supplements and

functional foods include *Ophiocordyceps sinensis* (Berk.), *Cordyceps militaris*, *Tolypocladium guangdongense*, and *Isaria cicadae* (Li et al. 2006a, Olatunji et al. 2018).

Originally, cordyceps was specifically referred to the species, *Ophiocordyceps sinensis* (formerly known as *Cordyceps sinensis*). However, due to its limited resources and high price, usage and yield of natural *O. sinensis* is diminishing during the past decade. Growth of *O. sinensis* is restricted to the Tibetan Plateau and its surrounding regions, including Tibet, Gansu, Qinghai, Sichuan, and Yunnan provinces in China and in certain areas of the southern flank of the Himalayas, in the countries of Bhutan, India (Uttarakhand) and Nepal, with 3,000 m as the lowest altitude for the distribution (Belwal et al. 2019, Li et al. 2011, Seth et al. 2014). Therefore, scientists have diverted their attention to the isolation of the active ingredients through large-scale production by fermentation. Apart from fermentation technology, scientists are also focusing on discovering alternative species having similar bioactivity.

Counterfeits of cordyceps frequently found in markets are, viz., *Stachys geobombycis*, *Stachys sieboldii*, *Lycopus lucidus* (Hsu et al. 2002, Li et al. 2006a). In addition, a number of closely related species are being sold in the market, labelled as, cordyceps (Zhang et al. 2020). Such intentional or unintentional introduction of cordyceps substitutes and counterfeits in the herbal market can affect its medicinal efficacy and/or might lead to poisoning (Wu et al. 1996, Doan et al. 2017). Nevertheless, some substitutes such as *C. militaris*, *T. guangdongensis* and *I. cicadae* containing similar therapeutic bioactives are well recognized and marketed in China and elsewhere (Dong et al. 2015).

Globally, there is a lack of clear regulation and quality control for cordyceps products which may raise questions on its authenticity and damage its reputation for its pharmaceutical and therapeutic properties with the presence of counterfeit products flooding the market. Thus, the development of quality control method for cordyceps products in a systematic manner is important. A number of bioactive have been identified from cordyceps, which include nucleosides (such as cordycepin and adenosine), polysaccharides, ergosterol and D-mannitol (earlier known as cordycepic acid) (Belwal et al. 2019). Some of these chemicals have been suggested as the “rational markers” for quality control purpose; however, standardization of methodologies for uniform quality standards are needed to prevent products of uneven quality in the market (Li et al. 2006a). Therefore, quality control of cordyceps and its substitute products for the identification of rational marker(s) is very important to discriminate between natural and cultured cordyceps and ensure their efficacy, safety and toxicity and rationalize the escalating price of these commercial products. The present review is focused on the key points of quality assurance/quality control (QA/QC) in cordyceps bioprocessing; importance of developing quality marker(s) for cordyceps with respect to the possible toxicity through contamination; progress and status of different quality markers to check their authenticity and strengths and limitations of analytical techniques available for quality check of cordyceps.

Quality assurance, quality control and regulation

The National Medical Products Administration (NMPA) formerly known as “State Food and Drug Administration (SFDA) of China” had issued a Pilot Program on using cordyceps in Health Foods (SFDA Bao Hua No. 225, 2012) in August 2012 (HKMB 2016), urging pilot enterprises to organize relevant pilots in accordance with requirements. China Food and Drug Administration (CFDA) revealed that since 2016, excessive arsenic contamination has been detected in *O. sinensis*. Subsequently, CFDA commanded all cordyceps functional foods pilot enterprises to stop their production on 26 February 2016. Thereafter, media reports claimed *O. sinensis* to be a poison rather than a functional food with pharmaceutical activity. Such media report impacted the health food market and the marketing of *O. sinensis* and its products.

There were earlier reports of high lead content in *O. sinensis* powder which was due to the practice of inserting Lead (Pb) bars inside the cordyceps to increase their weight (Liou et al. 1994, 1996, Wu et al. 1996). Blood Lead levels (BLL) in the Taiwanese adults were also found to be positively correlated with the history of taking lead-contaminated Chinese herbal drugs (Liou et al.

1994, 1996). Two cases of lead poisoning wherein one patient took the herbal medicine for 6 months and the other patient for more than 1 year (Wu et al. 1996). In total it was estimated that more than 2 g of Lead (Pb) was ingested by the patient. About 60 cases of cordyceps poisoning was reported from southern Vietnam between 2008 and 2015 (Doan et al. 2017). Ingestion of cicada flowers infected with the fungus *O. heteropoda* containing ibotenic acid, which is a powerful neurotoxin was found to be the cause of this poisoning. The neurotoxin exhibit symptoms ranging from dizziness, vomiting, salivation, jaw stiffness, urinary retention, seizures, hallucinations to even coma. Keeping in mind the safety and efficacy of *O. sinensis*, Zuo et al. (2013) studied six heavy metal (Mercury, Arsenic, Chromium, Cadmium, Copper and Lead) contents in natural *O. sinensis* and soil samples from fungal collection source by atomic absorption spectrometry (AAS) (Zuo et al. 2013). Cordyceps and soils collected from the same location were found to be contaminated with arsenic (As) and copper (Cu) which indicated contaminated soil used for growing cordyceps as the source of heavy metal contamination.

The elemental analysis using ICP-MS revealed that apart from essential elements, *O. sinensis* samples also contained a number of heavy metals such as Cd, Pb, and As. Lead (Pb) contents were found to be below 2.0 ppm in the analyzed samples which is below the set limit (5 ppm) for herbal medicines according to Pharmacopeia (Wei et al. 2017). However, the arsenic level in caterpillars of *O. sinensis* was significantly higher than in its mycelium which varied from 3.0-32 ppm possibly due to contamination of soil where they were grown. Zhou & et al. (2018) found higher Cu, Pb, Cd and Hg contents in stroma as compared to *O. sinensis* caterpillar when estimated using high performance liquid chromatography – inductively coupled plasma mass spectrometric (HPLC–ICP-MS) method (Zhou et al. 2018). However, arsenic was mainly found in the body of the caterpillar. Inorganic arsenic, only accounted for 8.69% of the total arsenic. They observed that the dietary exposure values of all the elements were below the safety limits assigned by “Joint FAO/WHO Expert Committee on Food Additives (JFCFA)” for these heavy metals.

As the popularity of cordyceps mushroom extract as health supplements for sports/energy/respiratory function/sexual health, is rising, so is the search by formulators for alternatives to provide similar benefits at a more affordable price. Artificially cultured cordyceps (solid fermentation technology) in laboratories is often grown on bedding materials such as sawdust, wood chips, compost or straw (Lin et al. 2017). Without conducting proper quality control of the raw materials, it is difficult to assess if the material has xenobiotic residues (pesticides, heavy metals). There is a possibility of these xenobiotics to be taken up by the fruiting body while growing and accumulate inside or remain in the bedding material which might be difficult to separate from the final product. Hence, low quality production methods can lead to unintentional exposure to toxic xenobiotics in the cordyceps. Sometimes, artificially cultured cordyceps are mycelium grown on rice flour or glutinous grain containing high levels of α -glucan as polysaccharides, instead of the naturally available active β -D-glucans having anti-cancer properties (Nammex 2021).

Quality assurance (QA) and quality control (QC) are the two pillars to ensure the quality of a product. Whereas Quality Assurance is primarily process oriented and focuses towards defect prevention, quality control is mainly product oriented and focuses on defect identification. Thus, it is important to establish quality check at the raw materials stage (such as the authentic cordyceps culture, substrates used to grow cordyceps and other chemicals needed to prepare the finished products) as well as the finished product stage to ensure that the customer is buying a product as claimed in the label of a finished product (Peterman & Žontar 2014). This can only be achieved by improvement and development in the quality test processes so that source of counterfeits or contaminants in the raw materials can be eliminated and product label claim established through QC of the finished product. Although it is important that the industry involved in cordyceps product manufacturing establish a good quality management system at every stage of the manufacturing process, the role of the regulatory body and testing facilities involved in checking the quality of the finished product and testing the label claim is also important to put a check on the spread of

adulterated products flooding the natural products market (Peterman & Žontar 2014). The various stages of cordyceps production and the quality checks required are presented in Fig. 1.

Bioprocessing of cordyceps includes broadly three sections: procurement of screened authentic isolates, raw materials and substrates; inoculation, incubation and harvest of the isolates for biomass production and downstream with formulation processing and packaging for storage/marketing of the formulated product. During the bioprocessing, quality checks at every stage should be followed. Initially, quality assurance should be applied for the identification of the isolates to be used for the entire manufacturing process through molecular marker technique, rDNA gene sequence. Chemical testing and verification of substrates and raw materials against the certificate of analysis should be determined for assuring the absence of heavy metals, pathogens and xenobiotics. Sometimes, organic substrates (like straw, husks, shells) can be contaminated with heavy metals, xenobiotics and pathogens (coliforms, *Staphylococcus* sp. etc.). Thus, the first bioprocessing step plays a crucial role for achieving quality of the finished products. It is important to prevent secondary microbial contamination during the entire bioprocess as the fermentation batch period is quite lengthy for cordyceps. During the downstream processing, it is again important to follow SOP/WI so that certificate of analysis could be generated and label claims verified (concentrations of active ingredients such as adenosine/cordycepin/mannitol/polysaccharides; material weight, moisture content, shelf life and absence of contaminants and pathogens) for the finished product. Other than QA/QC by the manufacturer, quality inspections taking random samples by national regulatory bodies are important to ensure that the products in the market are safe for consumption and are up to the standard specified by the licensing authority and in accordance with the product label (Peterman & Žontar 2014).

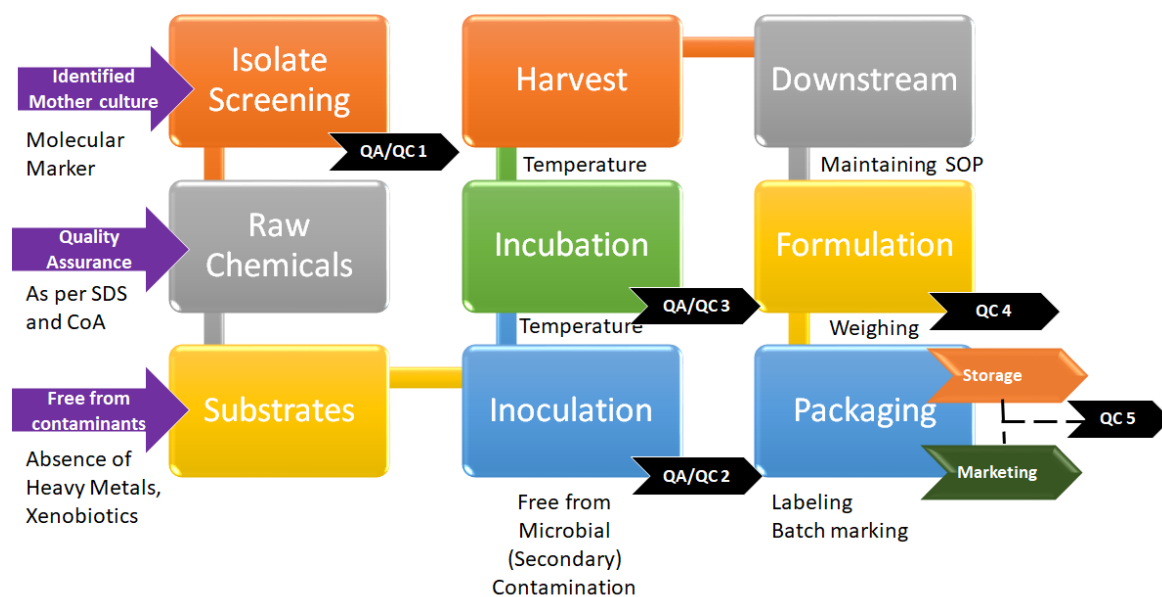


Fig. 1 – cordyceps bioprocessing and quality checks

Quality checks points

1. QA/QC 1: identification of the isolate through molecular marker. Chemical testing of substrate and raw materials (for example, water) for the absence of heavy metals and xenobiotic (SDS: safety Data Sheet; CoA: Certificate of Analysis)
2. QA/QC 2: prevention of secondary microbial contamination during batch process
3. QA/QC 3: Process control incubation temperature
4. QC 4: Quality check of the formulated product for active ingredients (for example, adenosine/cordycepin/mannitol/polysaccharide content as per label claim or specification) and absence of contaminants and pathogens.
5. QC 5: Quality check by national regulatory bodies or other agencies

Quality markers for cordyceps

Two valuable “cordyceps” species namely, *O. sinensis* (OS) and *C. militaris* (CM) contain many types of physiologically active substances such as nucleosides, polysaccharides, ergosterol and mannitol in varying proportion depending on their sources (Chen et al. 2013, Cui 2015, Das et al. 2010, Yue et al. 2013). These active ingredients are beneficial for human circulatory and respiratory system; our immune system; and hematogenic, cardiovascular, and glandular systems (Akaki et al. 2009, Zhou et al. 2014). *C. militaris* which can be easily cultured in both solid as well as liquid media are often used as a substitute for OS because of their similar chemical and pharmacological properties (Huang et al. 2009, Zheng et al. 2011, Dong et al. 2012).

The authentication of cordyceps remains a challenge. The quality markers proposed by various authors has broadly been based on morphology (Microscopy, Depth profiling by FT-IR), molecular (DNA sequencing or PCR based RAPD, SSCP, RFLP), chemical (protein, polysaccharides and precursors such as nucleosides and nucleotides) and metabolites (ergosterol, cordycepic acid) each having associated advantages and challenges (Li et al. 2006a). Table 1 provides a summary of quality markers proposed by various authors for distinguishing natural OS from cultured OS and CM.

Table 1 Summary of quality markers

S. No.	Quality marker	Type of cordyceps studied	Identification technique used	Reference
1.	Morphological marker			
1.1	Photoacoustic spectra of head, body, tail and leaf	Natural <i>O. sinensis</i>	Depth-profiling FT-IR photoacoustic spectroscopy	Du et al. (2017)
2.	Molecular marker			
2.1	Mutations on ITS2 region/ PCR-SSCP Profile	Natural & cultured <i>O. sinensis</i> Cultured <i>C. militaris</i>	PCR-SSCP	Kuo et al. (2006, 2008)
2.2	DNA sequence	Natural <i>O. sinensis</i>	ITS sequences and RAPD-SCAR	Lam et al. (2015)
2.3	DNA sequence analysis with identification of two restriction endonucleases	Natural <i>O. sinensis</i> Cultured <i>C. militaris</i>	PCR-RFLP followed by gel electrophoresis	Wei et al. (2016)
2.4	Primer pairs	Natural <i>O. sinensis</i>	PCR amplification using primer pair followed by gel electrophoresis	Li et al. (2019)
3.	Chemical markers			
3.1	Adenine, uracil, adenosine, guanosine, uridine and inosine and adenosine monophosphate (IS)	Natural & cultured <i>O. sinensis</i>	CE	Gong et al. (2004)
3.2	water-soluble profile (nucleosides)	Natural & cultured <i>O. sinensis</i>	CE	Li et al. (2004b)
3.3	Ergosterol, adenosine, cordycepin, cytidine, guanosine, thymidine, uridine, 2-deoxyuridine, adenine, cytosine, guanine, thymine and uracil	Natural & cultured <i>O. sinensis</i> Cultured <i>C. militaris</i>	RP-HPLC-DAD	Li et al. (2004a)
3.4	Water-soluble Protein profile	Natural <i>O. sinensis</i> Cultured <i>C. militaris</i>	CE	Takano et al. (2006)
3.5	Adenosine, cordycepin, cytidine, guanosine, inosine, thymidine, uridine, cytosine, guanine, thymine, and uracil	Natural & cultured <i>C. sinensis</i> Cultured <i>C. militaris</i>	RP-HPLC-DAD	Yu et al. (2006)

Table 1 Continued.

S. No.	Quality marker	Type of cordyceps studied	Identification technique used	Reference
3.6	Fingerprint analysis of nucleosides (Guanosine, hypoxanthin, uridine, adenosine, adenine, cordycepin).	Cultured <i>C. militaris</i>	HPLC-DAD	Yu et al. (2007)
3.7	Ergosterol and ergosteryl ester	Natural <i>O. sinensis</i>	HPLC-DAD	Yuan et al. (2007)
3.8	Uridine, inosine, guanosine, adenosine, and cordycepin	Natural <i>O. sinensis</i>	HPLC-DAD	Yang & Li (2009)
3.9	Adenine, adenosine, cytosine, cytidine, uracil, uridine, guanine, guanosine, hypoxanthin, inosine, thymine, thymidine, 2-deoxyuridine and cordycepin.	Cultured <i>O. sinensis</i> Cultured <i>C. militaris</i>	UPLC	Yang et al. (2007a)
3.10	Cytosine, uracil, uridine, hypoxanthine, 20-deoxyuridine, inosine, guanosine, thymidine, adenine, adenosine, and cordycepin	Natural & cultured <i>O. sinensis</i> Cultured <i>C. militaris</i>	CEC	Yang et al. (2007b)
3.11	Adenine, cytosine, guanine, hypoxanthine, thymine and uracil	Natural & cultured <i>O. sinensis</i> Cultured <i>C. militaris</i>	HPLC	Fan et al. (2007)
3.12	Adenosine, cordycepin, 2'-deoxyadenosine, guanosine and uridine	Natural & cultured <i>O. sinensis</i> Cultured <i>C. militaris</i>	HPLC-UV	Ikeda et al. (2008)
3.13	IR characteristic peaks	Natural & cultured <i>O. sinensis</i>	FTIR-2D-IR	Yang et al. (2009b)
3.14	Uracil, cordycepin, adenine, adenosine, uridine, hypoxanthine, inosine and guanosine, mannitol, glucose and trehalose and myriocin	Natural & cultured <i>O. sinensis</i> Cultured <i>C. militaris</i>	HPLC-DAD-ELSD	Wang et al. (2009)
3.15	Lauric acid, myristic acid, pentadecanoic acid, palmitoleic acid, palmitic acid, linoleic acid, oleic acid, stearic acid, docosanoic acid and lignoceric acid, ergosterol, cholesterol, campesterol and sitosterol	Natural & cultured <i>O. sinensis</i> Cultured <i>C. militaris</i>	GC-MSD	Yang et al. (2009a)
3.16	Rhamnose, ribose, arabinose, xylose, mannose, glucose, galactose, mannitol, fructose and sorbose	Natural & cultured <i>O. sinensis</i> Cultured <i>C. militaris</i>	GC-MSD	Guan et al. (2010)
3.17	Adenosine	Cultured <i>O. sinensis</i>	NIR	Xu et al. (2012)
3.18	Polysaccharides	Natural & cultured <i>O. sinensis</i> Cultured <i>C. militaris</i>	carbohydrase hydrolysis- HPSEC – DAD – ELSD or derivatization- HPLC-DAD–ESI-MS/MS	Guan et al. (2011)
3.19	Nucleotides, nucleosides and their transformation products	Natural & cultured <i>C. sinensis</i> Cultured <i>C. militaris</i>	IP-RP-LC–MS	Yang et al. (2010)
3.20	Adenosine, cytidine, guanosine, uridine, inosine, thymidine, adenine, cytosine, guanine, uracil, hypoxanthine and thymine, AMP, CMP, GMP, UMP, and TMP	Natural <i>O. sinensis</i>	HPLC-DAD	Zuo et al. (2013)

Table 1 Continued.

S. No.	Quality marker	Type of cordyceps studied	Identification technique used	Reference
3.21	Polysaccharides	Natural & cultured <i>O. sinensis</i> Cultured <i>C. militaris</i>	PACE and HPTLC	Wu et al. (2014)
3.22	cordycepin, adenosine, uridine, guanylic inosine and thymidine glucoside	Natural <i>O. sinensis</i>	HPLC-DAD	Shen et al. (2014)
3.23	cordycepin, mannitol, amino acids, cyclic peptides, and glycosides	Natural & cultured <i>O. sinensis</i>	HPLC-MS/MS (ESI-Triple quadrupole)	Hu et al. (2015)
3.24	Protein	Natural <i>O. sinensis</i>	2-D Electrophoresis	Chinese Patent No. CN104076082B (2016)
3.25	Volatile oils	Natural <i>O. sinensis</i>	GC-MS	Chinese Patent No. CN104730192B, (2015)
3.26	Polysaccharides	Natural <i>O. sinensis</i>	HP-GPC	Han (2017)
3.27	AMP, phenylalanine, uridine, hypoxanthine, inosine, guanine, guanosine, dAMP, adenosine, adenine and cordycepin	Natural <i>O. sinensis</i>	MC-LC coupled with HPLC-DAD-MS	Qian & Li (2017)
3.28	Uracil, uridine, adenine, guanosine, and Adenosine (internal standard)	Cultured <i>O. sinensis</i>	HPLC-DAD	Chen et al. 2018

Morphological marker

There are various macroscopic and microscopic studies available for differentiation of OS from other similar species. The common morphological and microscopic features for identifications are the transverse sections of stroma and larvae and surface sections of stroma at the macro- and micro-level for distinguishing *cordyceps*. The larval body of *O. sinensis* is densely covered with bristles of various lengths and the perithecia is semi-embedded at the surface of the fertile portion of the stroma. The key morphological features of the species that are found in the market are as follows (Liu et al. 2011).

1. With larva body

1.1 Larva body with stroma

1.1.1 With bristles on the surface of larva body

1.1.1.1 Abundant, fine, 20–40 mm in length – *O. sinensis*

1.1.1.2 Rare, gross, up to 100 mm in length – *C. barnesii*

1.1.2 Without bristles on the surface of larva body

1.1.2.1 Densely covered with brown or brownish-red hyphae – *C. liangshanensis*

1.1.2.2 Without hyphae, with fine reticular striations on the surface – *C. Gunnii*

1.2 Larva body without stroma – *C. gracilis*

2. Without larva body, but with stroma – *C. militaris*

A fairly new approach for morphological identification to characterize natural *O. sinensis* selected from China was studied by depth-profiling of their head, body, tail and leaf using FT-IR photoacoustic spectroscopy (Du et al. 2017). The photoacoustic spectra (PS) of head, body, tail and leaf were used as input of a probabilistic neural network (PNN) to correctly identify the source of OS. Therefore, depth-profiling by FT-IR-PS can prove to be a unique technique to identify and control the quality of OS (Du et al. 2017).

Molecular marker

Molecular marker techniques viz., internal transcribed spacer (ITS) sequences, random amplified polymorphic DNA (RAPD) and sequence characterized amplified region (SCAR) methods are used as a reliable marker for authentication of cordyceps at the species level. The ITS regions (ITS1 and ITS2) of rDNA are commonly used to examine phylogenetic positions or relationships at a species or interspecies level. These molecular techniques are independent from environmental influences or origin of cordyceps. These techniques can handle a high percentage of inter-specific sequence divergence and have already been successfully demonstrated in various studies (Kuo et al. 2006, 2008, Lam et al. 2015). It is observed that SCAR markers derived from the RAPD results provide quick authentication of cordyceps.

Wei et al. (2016) patented a method for quality control which comprised of DNA sequence analysis and finding out two restriction endonuclease sites to distinguish *O. sinensis* from *C. grasilis*, *C. militaris* and other related fungi and their products. Recently a kit based on PCR amplification with primer pair (CC-2F:5 '-ATTAAGTCGTGGAAATG-3' and CC-2R:5 '-GATCAGGAATAGTGGGA-3) has been patented [1] to identify *O. sinensis* (Li et al. 2019).

Chemical markers

Capillary electrophoresis

Hierarchical clustering analysis based on characteristic of 32 capillary electrophoresis (CE) peaks was used to conclude that adenosine and inosine could be used as chemical markers for natural and cultured *O. sinensis* (Gong et al. 2004). Six nucleosides and bases namely, adenine, uracil, adenosine, guanosine, uridine and inosine were used in the study and Adenosine monophosphate (AMP) was used as the internal standard. Natural and cultured *O. sinensis* were found in different clusters in hierarchical clustering analysis.

Similarly, Li et al. (2004b) also used hierarchical cluster analysis and capillary electrophoresis (CE) method for distinguishing natural cordyceps from cultured cordyceps and commercial products. Authors found that the cultured cordyceps contains high levels of adenosine, guanosine and uridine. Contrary to the previous study by Gong et al. (2004), this study found that few natural cordyceps samples did not show any presence of nucleosides and hence suggested that the profiles of water-soluble constituents of *O. sinensis* could well be used as fingerprints for the quality control of cordyceps instead of specific nucleosides (Li et al. 2004b).

The differences in the protein profiles of cordyceps, its substitutes and its variants viz., *O. sinensis*, *C. militaris*, *C. kyushuensis*, *C. tenuipes*, *I. cicadae* and *P. atypicola* were evaluated using capillary electrophoresis by Takano et al. (2006). *O. sinensis*, *C. kyushuensis* and *N. atypicola* showed similar peak clusters of protein whereas clusters of *C. militaris* were partly different from those of other cordyceps and *P. tenuipes* and *P. cicadae* showed lower peak clusters. Therefore, protein profiles of cordyceps could also be used as fingerprints for classification of cordyceps and its quality control. The Chinese Patent No. CN104076082B also identified ten types of proteins specific to *O. sinensis* for detecting genuine *O. sinensis* (Chinese Patent 2016).

Capillary Electro Chromatography

A hybrid technique between HPLC and CE, Capillary Electro Chromatography (CEC), has evolved as a technique with high selectivity and efficiency. optimization CEC method used for the determination of 11 nucleosides and nucleobases simultaneously in cordyceps using pressurized liquid extraction (PLE) and central composite design (CCD) (Yang et al. 2007b). Cultured cordyceps was found to contain higher uracil, uridine, guanosine, and adenosine content whereas natural *O. sinensis* had higher inosine content. Inosine is the major biochemical metabolite of adenosine. Although few samples of cultured *O. sinensis* contained Cordycepin; a nucleoside abundantly found in cultured *C. militaris*, but none of the natural *O. sinensis* contained the active ingredient.

High-performance liquid chromatography (HPLC)

Several authors preferred cordyceps specific nucleic acid bases and nucleosides as quality markers to identify and discriminate natural from the cultured cordyceps using HPLC (Chen et al. 2018, Fan et al. 2007, Ikeda et al. 2008, Li et al. 2004a, Shen et al. 2014, Yang et al. 2007a, 2010, Yu et al. 2006, 2007, Zuo et al. 2013). Natural and cultured cordyceps (*O. sinensis* and *C. militaris*) were differentiated through hierarchical clustering analysis of adenosine, cordycepin, and inosine (Yu et al. 2006). Hence, adenosine, cordycepin, and inosine were suggested as rational markers for discriminating natural from cultured cordyceps (Yu et al. 2006). Yu et al. (2007) developed and optimized an efficient and accurate fingerprint HPLC-DAD method for the quality control of cultured *C. militaris* with markers namely, guanosine, hypoxanthine, uridine, adenosine, adenine and cordycepin. The chromatographic fingerprint with similarity evaluation was used effectively to identify cultured CM and distinguish their origin. Yang & Li (2009) patented methods for extraction and quality characterization of *O. sinensis* by randomly comparing the sample with the content and proportion of any three of the components out of uridine, inosine, guanosine, adenosine, and cordycepin as detected in reference sample (Yang & Li 2009) [2]. Fan et al. (2007) optimized an acid hydrolysis method for the HPLC analysis of nucleobases. As reported in previous studies, total nucleobases in natural *O. sinensis* was found to be much lower when compared to cultured cordyceps (*O. sinensis* and *C. militaris*). Ikeda et al. (2008) chose adenosine, cordycepin, 2'-deoxyadenosine, guanosine and uridine for the authentication of cordyceps (*O. sinensis* and *C. militaris*) and its substitutes. The same authors concluded that the ratio of nucleosides to adenosine contents could be used as a marker for authentication and quality control of cordyceps. Shen et al. (2014) in their patent established a method for HPLC fingerprint of cordyceps fungus nucleoside components in different products through comparison of the fingerprints of the stromata, mycelia and encarpia of *O. sinensis* sample (Shen et al. 2014). Quantitative determination was performed on the basis of common fingerprint peaks. Zuo et al. (2013) observed during the analysis of natural *O. sinensis* that the storage conditions had an effect on the content of total nucleosides and individual nucleotides in natural *O. sinensis*, with fresh samples containing higher amount than stored samples. Chen et al. (2018) combined similar analysis (SA) and HCA and established a HPLC based fingerprint analysis with quantitative analysis of multi-components by single marker (QAMS) for differentiating and evaluating the quality of fermented *O. sinensis*. Uracil, uridine, adenine, guanosine, and adenosine (as internal reference substance) were chosen as the quality markers.

An HPLC-DAD-ELSD (Evaporative light scattering detector) method was developed by Wang et al. (2009) for the simultaneous estimation of nucleosides/nucleobases, carbohydrates and myriocin; an atypical amino acid, in different species of natural and cultured *O. sinensis*, *C. militaris* and *C. cicadae*. Unlike nucleosides, carbohydrates and myriocin have no UV absorptivity and hence cannot be detected by diode array detector (DAD). Though refractive index detector can be used for carbohydrate analysis, it is the least sensitive detector and cannot function under gradient elution. ELSD response is independent of optical characteristics of a sample and hence can be used for in series with DAD for the analysis of carbohydrates and myriocin. Similar to previous reports, nucleosides/bases such as uridine, adenosine and guanosine contents were found to be high in commercial cultured cordyceps, and cordycepin in *C. militaris*. The carbohydrate contents of mannitol and trehalose were found to be higher in natural *O. sinensis* and hence the authors (Wang et al. 2009) suggested carbohydrates as a useful marker for discriminating cordyceps which can be included in its quality control.

Commonly used in conjunction with MS/MS, UPLC has come up as a variant of HPLC due to its distinct advantage in terms of high-resolution, short analysis time and less consumption of organic solvent. Yang et al. (2007a) presented a fast UPLC method for the estimation of 14 nucleosides and bases to discriminate cultured *O. sinensis* from *C. militaris* and found that uridine, guanosine and adenosine, as the main components in cultured *O. sinensis* whereas cordycepin was found to be abundant in cultured *C. militaris* and in a few samples of cultured *O. sinensis*.

Therefore, UPLC can be used as a superior alternative to HPLC for the analysis of nucleosides and bases in a much shorter time.

Multi-column liquid chromatography system using reverse phase (RP) and size exclusion columns (SEC) in series was attempted by Qian & Li (2017) in order to simultaneously analyze nucleosides & sterols (RP column) and polysaccharides & proteins (SEC) as both the macro- (polysaccharides and proteins) and micro-molecules (nucleosides and sterols) are the bioactive components in cordyceps. However, they suggested that the limitation of longer run time of 90 minutes encountered during the separation can be overcome by the use of rapid LC columns.

Likewise, ergosterol and ergosteryl esters have also been simultaneously estimated by HPLC by Li et al. (2004a) and Yuan et al. (2007).

Liquid chromatography – mass spectrometry (LC–MS)

An ion-pairing reversed-phase liquid chromatography–mass spectrometry (IP-RP-LC–MS) method was developed for the estimation of nucleotides (UMP, AMP and GMP), nucleosides (adenosine, guanosine, uridine, inosine, cytidine, thymidine and cordycepin) and nucleobases, (adenine, guanine, uracil, hypoxanthine, cytosine and thymine) to distinguish natural and cultured *O. sinensis* and cultured *C. militaris* (Yang et al. 2010). Effects of sample preparation on the transformation of nucleotides and nucleosides revealed that (1) nucleotides namely, AMP, GMP and UMP degraded to their respective nucleosides namely adenosine, guanosine and uridine, which further degraded to their respective bases in natural *O. sinensis*, cultured *C. militaris* and lab-cultured *O. sinensis*; (2) oxidative deamination of adenosine could be the source of inosine in natural *O. sinensis* which is different from commercial cultured *O. sinensis* where high temperature process could be responsible which might have deactivated the enzymes, during the production.

In another study, a mixture of quality markers including cordycepin, D-mannitol, phenylalanine, Phe-o-glucose, cyclo-Gly-Pro, and cyclo-Ala-Leu-rhamnose was used to develop HPLC-MS/MS method to simultaneously identify and quantify them (Hu et al. 2015). High level of glycosidases activity in *O. sinensis* was indicated by the presence of two glycosides, namely, cyclo-Ala-Leu-rha and Phe-o-glu, in the natural *O. sinensis*, which were not found in the cultured substitutes.

Fourier-transform infrared spectroscopy (FTIR)

Cordyceps of different origins, capsule products and counterfeits were distinguished by a two-dimensional correlation infrared spectroscopy (2D-IR) (Yang et al. 2009b). Characteristic fingerprints in the range of 1400–1700 cm^{-1} and 2D spectra of 670–780 cm^{-1} ×1400–1700 cm^{-1} were used to discriminate cordyceps, commercial products and counterfeits. Pharmacopoeia of the People's Republic of China (2002) has set minimum value of $\geq 0.01\%$ for adenosine content in cordyceps, which can be an important criterion in the manufacture of cordyceps mycelium. Xu et al. (2012) patented a quick method for detecting adenosine content in *O. sinensis* mycelial powder using FT-IR in the near infra-red region at 4,902.49-4,817.64 cm^{-1} and 4,740.49-4,107.91 cm^{-1} .

Saccharide mapping

Guan et al. (2011) carried out saccharide mapping to distinguish between natural and cultured cordyceps (*O. sinensis* and *C. militaris*). Polysaccharides from most of the natural and cultured cordyceps had similar responses to carbohydrase hydrolysis followed by discrimination on the basis of HPLC profiles of pectinase hydrolysates in HPLC-ESI-MS/MS, which was helpful in the quality control of cordyceps. Mannose, glucose, and galactose were the common monosaccharides found in the enzymatic hydrolysates. Galacturonic acid, detected in pectinase hydrolysates from cultured cordyceps, was not detected in natural *O. sinensis*, which could be effectively used for discriminating cultured from natural cordyceps. Similarly, Wu et al (2014) also used saccharide mapping to differentiate natural from cultured cordyceps. The authors used partial acidic and/or enzymatic digestion (-amylase, -glucanase and pectinase) followed by analysis with carbohydrate gel electrophoresis (PACE) and high performance thin layer chromatography (HPTLC) to obtain

the profiles of the hydrolysates. Their results showed the presence of 1,4--d-glucosidic, 1,4--d-glucosidic and 1,4--d-galactosidic linkages in natural and cultured *O. sinensis*, cultured *C. militaris*, natural *O. sinensis*, *C. gracilis* and *I. cicadae*. Both cultured *C. militaris* and natural *O. sinensis* had similar polysaccharides, which suggests that *C. militaris* can be efficiently be used as a substitute of *O. sinensis*. A patent by Han (2017) carried out qualitative and quantitative analysis using size exclusion chromatography for the authentication of *O. sinensis* using polysaccharides corresponding to 200K – 2560K of pullulan series and 250K – 1200K of dextran series.

Gas Chromatography-Mass spectrometry (GC-MS)

Natural *O. sinensis*, *C. liangshanensis* and *C. gunnii*, as well as cultured *O. sinensis* and *C. militaris* were distinguished based on their free fatty acid and free sterol profiles by Yang et al. (2009a). The free fatty acids namely, lauric acid, myristic acid, pentadecanoic acid, palmitoleic acid, palmitic acid, linoleic acid, oleic acid, stearic acid, docosanoic acid and lignoceric acid and free sterols namely, ergosterol, cholesterol, campesterol and sitosterol were determined using GC–MS. The extractions of the samples were done using pressurized liquid extraction (PLE) followed by trimethylsilyl (TMS) derivatization. Ergosterol, palmitic-, linoleic-, oleic- and stearic acids were found to be the main components of both natural and cultured cordyceps. Hierarchical clustering analysis could discriminate natural cordyceps having high contents of palmitic- and oleic acids in comparison to cultured ones. GC-MSD method has also been employed to analyze monosaccharides, (rhamnose, ribose, arabinose, xylose, mannose, glucose, galactose, mannitol, fructose and sorbose) by Guan et al. (2010). They also used stepwise PLE for extraction followed by acid hydrolysis and derivatization prior GC-MS. Natural *O. sinensis* was found to contain > 7.99% free mannitol compared to cultured *O. sinensis* and *C. militaris* (5.83%). Another study (Chinese Patent 2015) attempted to identify *O. sinensis* on the basis of their volatile components using NIST mass spectral library as reference.

Stable carbon isotope analysis

The $\delta^{13}\text{C}$ profiles of *O. sinensis* collected from 5 representative different habitats were studied by Guo et al. (2017). The $\delta^{13}\text{C}$ was shown to be maximum at the head, with a slight decrease from the head to the end of thorax and maintenance of lower $\delta^{13}\text{C}$ values in the rest parts of abdomen of *O. sinensis*. The growth stages of *O. sinensis* can be speculated depending on this data as the symptom-free, symptom-appearing, and stroma-germinating stages.

Importance of different quality markers

Nucleic acid based markers

Nucleic acid-related compounds such as adenosine, guanosine, uridine, adenine, cordycepin and uracil are few of the major active components of *Cordyceps sensu lato* known to exhibit pharmacological activities. Adenosine can treat supraventricular tachycardia and inhibit the release of neurotransmitters in the Central Nervous System (Li et al. 2006a). Cultured cordyceps are known to contain higher level of nucleosides as compared to fresh natural *O. sinensis*. However, nucleoside content increases in the dried or processed natural *O. sinensis* forms. Cordycepin (3'-deoxyadenosine), a derivative of adenosine, exhibits anti-tumour, insecticidal and anti-bacterial activity (Paterson 2008). Cordycepin, which is primarily found in *C. militaris* in high content, is also present in in *O. sinensis* and *C. kyushuensis* as well (Das et al. 2010). The same was observed by Ikeda who found that the contents of adenosine (2.44–14.15 mg g⁻¹), guanosine (2.96–14.79 mg g⁻¹) and uridine (2.00–20.29 mg g⁻¹) were higher in cultured *O. sinensis* than in natural *O. sinensis* (fruiting bodies or caterpillars) (Ikeda et al. 2008). The contents of cordycepin (3.33- 6.36 mg g⁻¹) in cultured *C. militaris* were more than 100 times higher than in cultured *O. sinensis* (0.043 mg g⁻¹), which further supported previous studies by Fan et al. (2007) and Li et al. (2004a). Except for cordycepin, contents of the other three nucleosides were lower in cultured *C. militaris* (adenosine, ≤ 1.58 mg g⁻¹; guanosine, 0.68 mg g⁻¹; uridine, 1.53 mg g⁻¹) compared to cultured *O. sinensis*,

suggesting that cordycepin could be a chosen marker for *C. militaris*. Further, Li et al. (2002) reported that inosine, a major biological metabolite of adenosine, and cordycepin, were characteristic of both natural *O. sinensis* and *C. militaris*. In addition, the ratio of nucleosides to adenosine contents in cordyceps can also be a useful marker for authentication and quality control of cordyceps as suggested by Ikeda et al. (2008). Hence, nucleosides and nucleobases namely, adenosine, inosine, guanosine, uridine and cordycepin could be preferred candidates as quality markers (Table 2). Moreover, the nucleosides can be both qualitatively and quantitatively analyzed using a variety of techniques namely, HPLC, CE, LC-MS/MS and IR.

Polysaccharides

Polysaccharides, which are responsible for immunopotential, hypoglycemic, antioxidant, and antitumor activities are present in the range of 3 to 8% of the total dry weight in cordyceps and can be considered as marker for quality control of cordyceps (Table 2) (Li et al. 2001, 2002, 2003, 2006b, Leung et al. 2009, Zhu et al. 2009, Lee et al. 2010, Li et al. 2013). Polysaccharides such as CSP-1, isolated from cultured cordyceps has shown antioxidant activity (Kiho et al. 1999); CPS-1, isolated from cultured CM has shown anti-inflammatory activity; and four other polysaccharides named CPS-2, CPS-3, CPS-4 & CPS-5 has also been isolated from cultured CM (Yu et al. 2004).

However, analysis of polysaccharides is a challenge because of their complex structure and no absorptivity in the UV range. Polysaccharides in cordyceps (natural and cultured) have been isolated on the basis of their molecular mass using gel permeation chromatography (GPC) (Han 2017). The molecular distribution pattern of the water extract of the polysaccharides (pullulan series and dextran series) from a few authentic OS samples was compared using size exclusion chromatography (SEC). Neither other cordyceps species nor fake samples contained CSP polysaccharide marker. The QC marker was further isolated and used as a reference chemical in GPC quantitative analysis, which enabled evaluation of not only true/false authentication but also the quality of *O. sinensis* samples. Quality control laboratory for analysis of cordyceps samples which would include the polysaccharide marker, a HPGPC column, and software for data analysis have the potential to be converted as a commercial kit. Recently, “saccharide mapping,” which involves carbohydrase hydrolysis followed by chromatographic analysis (HPLC or HPTLC), has been established for qualitative analysis of polysaccharides by Guan et al. (2011) and Wu et al. (2014). The samples could be either analyzed by HPSEC–DAD–ELSD technique or derivatized with 1-Phenyl-3-methyl-5-pyrazolone (PMP) prior to HPLC–DAD–MS analysis (Guan et al. 2011). However, separation of polysaccharides and the hydrolysates simultaneously in HPSEC is difficult. In addition, the sensitivity of evaporative light scattering detector (ELSD) and refractive index detectors (RID), commonly used for polysaccharides, is poor (Li et al. 2013). Carbohydrate gel electrophoresis (PACE), which has good sensitivity, high resolution and high throughput could be an alternative although its resolution for the analysis of different monosaccharides is poor due to their 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) derivatives (Goubet et al. 2002, Wu et al. 2013). High-performance thin-layer chromatography (HPTLC) could be another alternative for separation of different types of monosaccharides (Xie et al. 2012). Combination of HPTLC and PACE to understand comprehensive profile of enzymatic and partial acid hydrolysates of polysaccharides was used by Wu et al. (2014). Saccharide mapping of different species of cordyceps, including *O. sinensis*, *C. militaris*, *C. gunnii*, *C. liangshanensis*, *C. gracilis*, *C. hawkesii* and *C. cicadae* were estimated based on HPTLC and PACE, which provided better understanding of the structural characteristics of polysaccharides in these species of cordyceps.

D – Mannitol

D-mannitol, commonly known as cordycepic acid, contributing to more than 3.4% of the total dry weight in natural OS is used to reduce raised intracranial pressure, used as laxative, and shown to exhibit antitussive, diuretic, and anti-free radical activities. Hence, mannitol also can be considered as marker of cordyceps for its quality control (Table 2) (Li et al. 2006a, Lin & Li 2011, Hu et al. 2015).

Since mannitol is a carbohydrate, it has no UV absorptivity; hence cannot be analyzed with HPLC-UV detector. P-nitrobenzoyl derivatization prior analysis with UV detector is an option, although it increases the complexity of sample preparation (Schwarzenbach 1977). Low sensitivity Refractive index (RI) detector in conjunction with HPLC has also been used previously (Li et al. 2006a). However, to improve the resolution and enrich the sample, solid phase extraction as a cleanup step was used prior detection with RI. The evaporative light scattering detector (ELSD) in place of RI detector is another option to detect mannitol (Li et al. 2006a). Alternatively, ESI-MS/MS can be a better alternative which was the method of choice by Hu et al. (2015).

Ergosterol

Ergosterol is a sterol and precursor of vitamin D2, which gets converted to D2 through chemical reaction in presence of UV light. It has significant pharmacological activities (Bok et al. 1999, Slominski et al. 2005). Ergosterol analogues of CM have anti-viral, antiarrhythmic and suppression of Berger's disease properties (Li et al. 2006a, Yue et al. 2013). Free ergosterol contributes to a variety of cellular functions. The fruiting bodies of cordyceps have higher ergosterol content (10.68 mg/g) than their mycelia (1.44 mg/g) (Yue et al. 2013). Hence, ergosterol can be a useful chemical marker for evaluating the quality of *O. sinensis* (Table 2). Ergosterol can be easily analyzed by HPLC although the samples need prior saponification for estimating ergosteryl esters by HPLC analysis (Li et al. 2004a, Yuan et al. 2007).

Analytical techniques for quality markers

Varied methods such as microscopic, DNA approaches using PCR technology, capillary electrophoresis (CE), FT-IR, FT-IR photoacoustic spectroscopy, HPTLC, HPLC, GC-MS and LC-MS/MS have been studied in an attempt to authenticate cordyceps as there exists a need for its quality control and authentication method. A quality control method for authentication ideally should be (a) able to differentiate authentic samples from their substitutes/counterfeits using significant quality marker(s) (b) rapid and easy to operate; (c) relatively cheap and low cost of maintenance; (d) simple and do not require specialized trained manpower to operate (devoid of complicated operation); (e) repeatable and reproducible; (f) applicable for both qualitative and quantitative analysis; (g) reliable with a capacity to handle large number of sample batches; (h) practical for commercial application and easily available in QC laboratories and (i) capable of multi-component analysis in a single operation (Li et al. 2006a).

Table 2 Rational quality markers of cordyceps

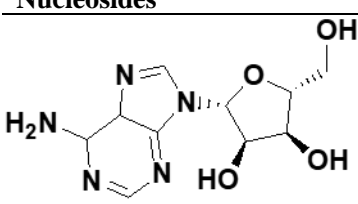
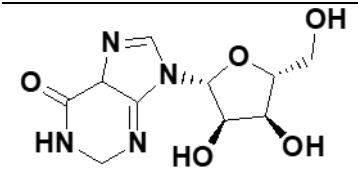
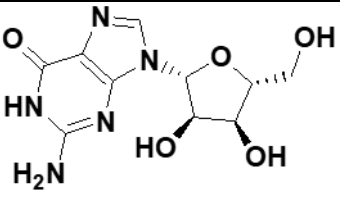
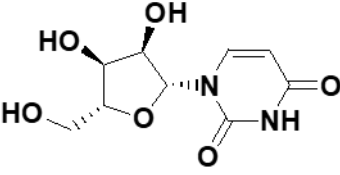
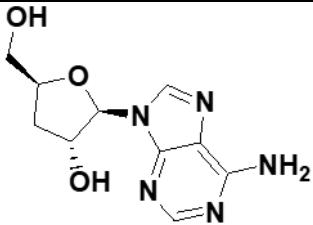
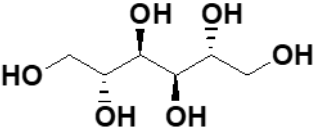
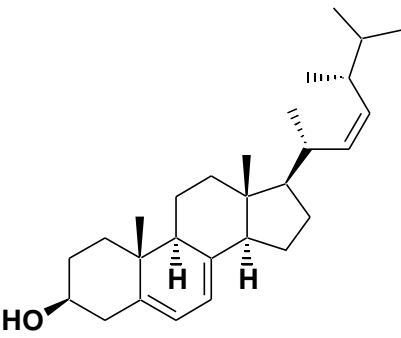
Quality Marker of choice	General/specific Pharmacological activity	Type of cordyceps	Analytical method of choice	Suggested by
Nucleosides 	Depress the excitability of CNS neurons and inhibit release of various neurotransmitters presynaptically, anticonvulsant, treat chronic heart failure	Higher in cultured <i>O. sinensis</i> (2.44–14.15 mg/g) than natural OS or cultured <i>C. militaris</i>	HPLC, LC-MS	Ikeda et al. (2008)
Adenosine 	Stimulate axon growth <i>in vitro</i> and in the adult central nerve system	Higher in natural <i>O. sinensis</i>	HPLC, LC-MS	Ikeda et al. (2008)
Inosine				

Table 2 Continued.

Quality Marker of choice	General/specific Pharmacological activity	Type of cordyceps	Analytical method of choice	Suggested by
 <p>Guanosine</p>	Regulation and modulation of various physiological processes in the nervous system	Higher in cultured <i>O. sinensis</i> (2.96–14.79 mg/g) than natural OS or cultured <i>C. militaris</i>	HPLC, LC-MS	Ikeda et al. (2008)
 <p>Uridine</p>	Regulation and modulation of various physiological processes in the nervous system	Higher in cultured <i>O. sinensis</i> (2.00–20.29 mg/g) than natural OS or cultured <i>C. militaris</i>	HPLC, LC-MS	Ikeda et al. (2008)
 <p>Cordycepin</p>	Anti-tumour, insecticidal and anti-bacterial activity	Higher in cultured <i>C. militaris</i> (3.33–6.36 mg/g). Mostly absent in natural <i>O. sinensis</i>	HPLC, LC-MS	Das et al. (2010), Ikeda et al. (2008)
 <p>D-mannitol</p>	Diuretic, antitussive, and anti-free radical activities	Higher in natural <i>O. sinensis</i> (3.4% of the total dry weight)	HPLC-ELSD, LC-MS/MS	Hu et al. (2015)
Polysaccharides (CSP-1 isolated from cultured <i>O. sinensis</i> and CPS-1,2,3,4,5 isolated from cultured <i>C. militaris</i>)	Anti-oxidant, immunopotential, antitumor and hypoglycemic activities	High in natural <i>O. sinensis</i> (3–8 % of total dry weight)	HPTLC and PACE, HPLC-MS/ELSD	Guan et al. (2011), Kihoh et al. (1999), Wu et al. (2014), Xie et al. (2012), Yu et al. (2004)
 <p>Ergosterol</p>	Cytotoxic activity, anti-viral activity, and anti-arrhythmia effect	Higher in natural <i>O. sinensis</i>	GC-MSD, HPLC-DAD	Yuan et al. (2007), Yang et al. (2009a)

Strengths and limitations

Morphological approaches

The conventional methods such as microscopic examination to identify morphological markers depend on the experience of botanical experts and also involve subjective judgement. Moreover, morphology of an organism is not uniform across environmental variations.

DNA-based approaches using PCR

In the perspective of economic feasibility, many manufacturers sell variants and substitutes as cordyceps. The substitutes, counterfeits and adulterants of *O. sinensis* can easily influence clinical importance of this medicinal herb. To guarantee a persistent adequacy and safety of cordyceps, a long-term quality control approach should be implemented for the confirmation of the species to be utilized as the source material. Apart from ecological impacts or origin of crude material, the use of DNA sequencing or molecular genetic methods using PCR technique can supplement the quality control parameters. Moreover, some useful identification tools are restriction fragment length polymorphism (RFLP) (Wei et al. 2016), random amplified polymorphic DNA (RAPD) (Chen et al. 1999), amplified fragment-length polymorphism (AFLP) (Savelkoul et al. 1999, van der Wurff et al. 2000), and direct rDNA sequencing of cordyceps. Internal transcribed spacer (ITS) sequences and the random amplified polymorphic DNA (RAPD)-sequence characterized amplified region (SCAR) are developed for ensuring the authenticated result (Kuo et al. 2006, 2008, Lam et al. 2015). These ITS sequences and the RAPD-SCAR marker enabled discrimination of OS from its common adulterants, including *O. gracilis*, *C. hawkesii* and *Drechmeria gunnii* as they are the routine markers used in evolutionary and diversity analysis at different phylogenetic characterization and identification. RAPD-SCAR method provides a quicker and user-friendly tool for the verification of cordyceps. RFLP, PCR-SSCP will be a valuable tool for rapid identification, even for large samples. Degenerate primer pairs (CITS-F10'/ CITS-R10'-2 and COI-F/COI-R) targeting the ITS region (113 bp) of *O. sinensis* for the COI gene (302bp) were successfully amplified from 17 Chinese cordyceps samples and its species-specific host were successfully designed recently by Zhang et al. (2020). This duplex PCR assay for Chinese cordyceps was successfully established for commercial samples which were tested for further verification. This duplex PCR method could be reliably used to identify Chinese cordyceps. It provides a new simple way to discern true commercial Chinese cordyceps from counterfeits in the marketplace. This is an important step toward achieving an authentication method and quality control for the Chinese medicine. The availability of genetic sequences could allow the development of technological devices, such as gene chips or specific kits to be enforced in routine quality control (Li et al. 2019). This DNA analysis is not affected by age, physiological conditions, environmental factors, as well as the methods of harvest, storage and processing.

Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR spectroscopy utilizing near-infrared (NIR) and mid-infrared (MIR) ranges, is a promising technique for the identification of TCM. It is a relatively fast technique for the qualitative analysis of complicated mixtures with minimal (KBr pellet) or no requirement for pre-treatment. Though reflectance spectroscopic method is used for quantitative analysis, it heavily depends on the sample size and its surface as the information is collected from the surface of a sample. Rough surface and strong heterogeneity in depths in OS samples may lead to failure in its identification. In depth-profiling FT-IR-photoacoustic spectroscopy (FTIR-PAS), local warming of sample followed by pressure fluctuations due to collisions with other molecules is detected by a very sensitive microphone. The technique receives abundant information in comparison to transmittance or reflectance spectra due to its depth profiling feature. The sample concentration is proportional to the detected signal. Quantitation is possible using multivariate statistical analysis, namely, artificial neural networks (ANN) and partial least square (PLS) (Du et al. 2017).

With 2D-IR, carried out with a conventional spectrometer and external perturbation (e.g. electrical, thermal or acoustic excitation, etc.), one can obtain desired 2D-IR correlation spectra. Since the peaks are spread over the second dimension, overlapped peaks are resolved. 2D-IR in combination with secondary derivative IR spectroscopy is applied in analysis and discrimination of cordyceps and other TCM. In 2D-IR technique, Yang et al. (2009b) observed that the two counterfeits (*Stachys geobombycis* and *Stachys sieboldii*) had almost all the characteristic peaks like real *Cordyceps sensu lato*, with weak intensities of some peaks. Therefore, the technique needs additional features and in depth interpretation which might pose difficulty in analyzing large sample size in a quality control laboratory.

Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS is a unique, highly sensitive, low operational cost and versatile technique suitable for volatile compounds. It needs moderate sample preparation involving solvent extraction and concentration and very low sample volume for injection. For nonvolatile components, the sample requires derivatization prior analysis (Falaki 2019). GC-MS techniques was used to identify the chemical composition of the essential oil of *O. sinensis* (Chinese Patent 2015). [3] The result identified 38 volatile components through comparison with NIST library data. Free fatty acids and free sterols have also identified with GC-MS (Yang et al. 2009a). However, prior derivatization was necessary to detect the sterols using GC-MS. Since, most of the active components of cordyceps, namely, nucleosides, mannitol, ergosterols are non-volatile in nature; GC-MS cannot be a method of choice as additional derivatization with expensive derivatizing agents such as BSTFA would be essential (Falaki 2019).

Capillary electrophoresis (CE)

Capillary electrophoresis is a rapid technique requiring low sample volume and short pretreatment. Narrow capillaries used in CE helps to reduce band-broadening as experienced in high performance liquid chromatography (HPLC) which in turn renders good resolution to the technique. However, changes in pH directly affect the molecular charge and flow in CE; thus variations in pH can have a greater impact on CE as compared to HPLC. In addition, pH can be affected by the temperature as well. Compared to CE; CEC is a hybrid technique between HPLC and CE with high selectivity and efficiency, although both CE and CEC are not readily available instruments in a QC lab (Takano et al. 2006). Several groups mostly prior 2010 have used CE or CEC (a hybrid technique between HPLC and CE); techniques to distinguish cordyceps from their substitutes on the basis of nucleosides and water soluble protein profiles (Gong et al. 2004, Li et al. 2004b, Takano et al. 2006, Yang et al. 2007b). Li et al. (2004b) successfully utilized CE water soluble constituent profile (nucleoside) to distinguish natural *O. sinensis* from cultured OS and commercial products. classification of *Cordyceps*, *Paecilomyces*, and *Nomuraea*, on the basis of their protein profiles by Capillary electrophoresis (CE).

High performance liquid chromatography (HPLC)

By far the most commonly employed technique for quality assessment of cordyceps has been HPLC and its variants (Size exclusion, UPLC) due to its versatility, ready availability in any quality control laboratory, appropriate for routine analysis, the ease of operation and its suitability in analyzing a variety of nonvolatile, thermo-labile quality markers present in cordyceps (nucleosides, nucleobases, nucleotides, ergosterol, mannitol and polysaccharides) (Chen et al. 2018, Fan et al. 2007, Ikeda et al. 2008, Li et al. 2004a, Shen et al. 2014, Yang et al. 2007a, 2010, Yu et al. 2006, 2007, Zuo et al. 2013). HPLC is a convenient method for analysis of a wide variety of chemicals which are otherwise not amenable to GC. The variety of columns (C18, size exclusion, cation exchange, amino, phenyl) in variable sizes and detectors (UV, PDA, RI, fluorescence, ELSD, MS) offered by HPLC renders versatility to detect varied types of markers. Out of the available detectors, UV/PDA detector can be used for most of the analytes namely, nucleosides, nucleobases, nucleotides, water soluble proteins, amino acids, ergosterol and free sugars.

Polysaccharides can either be analyzed by RI detector or ELSD. The evaporative light scattering detector (ELSD), a replacement of RI is a destructive detector commonly used for analysis of compounds that do not absorb UV radiation, such as polysaccharides, sugars, fatty acids, lipids, oils, phospholipids, and triglycerides. ELSD has been successfully used for the analysis of polysaccharides by Wang et al. (2009) and Guan et al. (2011). Cordycepic acid or mannitol, which cannot be detected by UV detector, can also be analyzed using HPLC–ELSD method (Wang et al. 2009). Mass spectrometer detector in conjunction with HPLC or UPLC such as ESI-MS/MS has also been successfully employed for the detection of a wide range of markers namely, nucleosides, nucleobases, nucleotides, ergosterol, mannitol and polysaccharides amino acids, cyclic peptides and glycosides (Guan et al. 2011, Hu et al. 2015 and Qian & Li 2017).

Conclusion

The emerging market demand for cordyceps as health supplements and pharmaceutical ingredient has put constraints on the demand and supply chain balance of naturally occurring cordyceps, or more so *O. sinensis*. This in turn has increased the possibility of introducing adulterated or counterfeits in the market as authentic cordyceps, thus compromising the quality of the finished product. Alternative approaches of biomass production in liquid fermenter using substitute; *C. militaris*, which has similar pharmacological activities, is also an option. Thus, it is essential that the quality of the product is maintained at every stage of the bioprocess, beginning with the right choice of the fungus through morphological and molecular techniques to instill confidence in the consumer. Bringing transparency by developing and following universal standardized quality protocols throughout the bioprocess of cordyceps will allow knowledge dissemination to the consumer through detailed label claims in the product. Introducing universal morphological, chemical and molecular rational markers will be convenient not only to manufacturers but also strengthen regulatory agencies to inspect local, imported and export products. Creating gene bank for preserving the fungal diversity can serve as a reference hub for knowledge dissemination globally.

Several authors have worked with different morphological, molecular and chemical markers in an attempt to find out the most suitable quality marker for cordyceps. Molecular markers are useful at the beginning of a bioprocess during screening and identification of authentic isolates; chemical markers have their distinct role for verifying the chemical constituents claimed in a product label. The most rational chemical markers should ideally be chosen based on the pharmacological properties it exhibits. In addition, the chosen markers should be fairly easily analyzed in a quality control laboratory using a suitable analytical technique. As has been prescribed by the “Pharmacopoeia of the People’s Republic of China (2002)” that adenosine in cordyceps should be $\geq 0.01\%$, this can be taken as a starting point for the quality control marker in the manufacture of cordyceps. Based on the previous studies, it can be recommended that nucleosides namely, adenosine, inosine, guanosine, uridine and cordycepin; mannitol, among carbohydrates, polysaccharides and ergosterol can be chosen as rational markers for cordyceps (Table 2). The technique of choice could be HPLC coupled with UV/PDA and ELS detector or LC-MS/MS for the identification and quantification of the markers. In addition to this, quick identification kits based on molecular primers pairs (true/false) as developed by Li et al. (2019) to identify *O. sinensis* can be employed for their onsite inspection. NIR based technique developed for analyzing and quantifying adenosine content [4] can be extrapolated to a hand-held NIR device for a quick onsite inspection (Zhang & Wei 2012).

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

DS and PD contributed conception and content of the review paper; PD wrote the

morphological and molecular marker and bioprocessing sections and the rest were written by DS. All authors contributed to manuscript revision, read and approved the submitted version.

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