Potential of Complementary Methods for the Authentication of Herbal Substances and their Mixtures

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Contents

Abbreviations:

°C	degree Celsius
1D	one dimensional
^{1}H	proton
A	adenosine
AA	aristolochic acids
DMHS	defined mixture(s) of herbal substances
BLAST	Basic Local Alignment Search Tool
C	cytosine
CBOL	Consortium for the Barcode of Life
$CDCL_3$	deuterated chloroform
Cq	quantification cycle
DCM	dichloromethane
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
EC	European Commission
EDQM	European Directorate for the Quality of Medicines & Health Care
EMA	European Medicines Agency
EtOH	ethanol
ETS	external transcribed spacer
FRET	fluorescent resonance energy transfer
G	guanine
GABA	γ-aminobutyric acid
GC	gas chromatography
GPB	botanical gardens of the Pharmaceutical Biology
HMP	herbal medicinal product
HMPC	Committee on Herbal Medicinal Products
HPLC	high performance liquid chromatography
IGS	intergenic spacer
IR	infrared
ITS	internal transcribed spacer
LB	Luria Bertani medium
MS	mass spectrometry

mtDNA	mitochondrial DNA
NMR	nuclear magnetic resonance
nrDNA	nuclear ribosomal DNA
NTS	non-transcribed spacer
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
Ph. Eur.	European Pharmacopoeia
qPCR	quantitative polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
s	seconds
T	thymin
TCM	Traditional Chinese Medicine
TLC	thin-layer chromatography
GUP	gardens of useful plants University Bonn
UV	ultraviolet
YT	bacto yeast / bacto tryptone-medium

1 Introduction

1.1 Medicinal plants

Formerly, the prevention and treatment of illnesses was mainly based on plants. The earliest written proof of the usage of medicinal plants for the preparation of herbal medicines was found on a Sumerian clay slab from Nagpur, dated about 5000 years ago (Petrovska, 2012). Due to traditions, customs and the newly recognized significance of alternative healing methods, but also as a result of higher costs or limited availability of conventional medicine in developing countries, plant derived medicine is still of great significance for healthcare worldwide.

A considerable part of the world population, e.g. almost 80 % of African and Asian people, rely on traditional medicine, mainly based on herbal sources, for their primary healthcare (Akerele, 1993; Sahoo et al., 2010). In Europe, herbal medicines nowadays not only are derived from European systems but also from many different traditions like Chinese, Indian, North and South American and African traditions (Shaw et al., 2012). There is a great diversity regarding the usage and also legislation of herbal medicines worldwide, entailing implications for safety of herbal substances. As a result, there is an increased demand for harmonization and globalization of standards for quality control for herbal substances coming from different cultures.

According to Directive 2001/83/EC, herbal substances are “all mainly whole, fragmented or cut plants, plant parts, algae, fungi, lichen in an unprocessed, usually dried, form, but sometimes fresh.” (Synonym: herbal drug (European Pharmacopoeia)). A herbal medicinal product is defined as “any medicinal product, exclusively containing as active ingredients one or more herbal substances or one or more herbal preparations, or one or more such herbal substances in combination with one or more such herbal preparations.”

1.2 Quality assessment, authenticity and safety of herbal medicinal products

Before accessing the market, quality, safety and efficacy of herbal medicinal products has to be evaluated. The standards and requirements for the quality assessment are generally described in regional pharmacopeias and regulatory guidelines. Thus, the regulation for standards on quality control is not consistent across countries (Fan et al., 2012; Sahoo et al., 2010). Discrepancies begin with basic questions like appropriate nomenclature for herbal substances and are continuing in demands on methods used for the evaluation of the identity (Shaw et al., 2012). In Europe, the European Pharmacopoeia (Ph. Eur.), released by the European Directorate for the Quality of Medicines and HealthCare (EDQM), defines transnational, congruent requirements on quality control for herbal substances and herbal preparations (Sahoo et al., 2010). The Ph. Eur. provides information on methods for quality control of medicines during development, production and marketing processes (Bauer and Franz, 2010a; Vlietinck et al., 2009) The Committee on Herbal Medicinal Products (HMPC) of the European Medicines Agency (EMA) aims at contributing to the harmonization of procedures and provisions regarding herbal medicinal products in the EU member states and publishes herbal monographs (van Galen, 2014; Qu et al., 2014).

The HMPC evaluates scientific data on safety and efficacy of herbal substances and herbal preparations and depending on experience with medicinal use, herbal medicinal products (HMP) are classified either as well-established use or traditional herbal medicinal product through Directive 2001/83/EC (Knöss and Chinou, 2012). Methods recommended in the Ph. Eur. for authentication of herbal medicines are mainly based on chemical analyses like TLC-, HPLC- or GC-fingerprints and morphological identification (macroscopic and microscopic). Mostly, quality assessment for chemical characterization is including an assay of at least one marker compound.

Intentional or accidental adulterations with other plants (Chan, 2009, 2011; Rueda et al., 2012; Vanherweghem et al., 1993), toxic heavy metals (Chan, 2003), pesticide residues (Xue et al., 2008) and mycotoxins, like e.g. aflatoxins (Gnonlonfin et al., 2013) or adulterations with synthetic drugs (Lilja et al., 2008), are main issues for quality

control of HMPs. Furthermore, incorrect processing methods may have implications on safety of herbal substances (Singhuber et al., 2009).

In this thesis, methods have been tested and developed, which should enable the identification of plants in mixtures of unknown composition. Several serious case reports arose from the misuse of herbal medicines. Such as, for instance, the use of Aristolochiae fangchi radix (Guang fangji) in a slimming tea in a medical clinic in Belgium, which led to interstitial renal fibrosis, in some cases progressing to terminal renal failure, in women who had consumed the slimming tea (Vanherweghem et al., 1993). Aristolochiae fangchi radix was taken accidentally due to a confusion of this herbal substance with Stephaniae tetrandae radix (Fangji), as both share a similar Chinese common herb name (Guang fangji and Fangji). Misuse of Aconite species, based on accidental confusion of toxic *Aconitum napellus* (Europe, North America) or inadequate processing of Aconiti radix preparata (*A. carmichaeli,* China) or Aconiti kusnezoffii radix preparata (*A. kusnezoffii;* China) was the reason for a multitude of poisoning incidents due to highly neurotoxic and cardiotoxic aconite alkaloids like aconitine or mesaconitine (Chan, 2009; Singhuber et al., 2009; Wang et al., 2009). Plants from the Aristolochiaceae family produce aristolochic acids (AA), whereof the highest amount of AA can be found in Aristolochiae manshuriensis caulis (Chan et al., 2006). Prolonged intake of this herb can lead to severe renal failure requiring dialysis or even kidney transplantation (Vanherweghem et al., 1993). Moreover, the consumption of AA is associated with the development of urothelial carcinoma of the upper urinary tract (Poon et al., 2007). AA is amongst the 2 % of the most potent carcinogens (Gold and Zeiger, 1996).

A recent case emphasized the necessity of quality control of herbal substances. A massive adulteration of Bupleuri radix (Bupleurum chinense) with Aristolochiae manshuriensis radix (*A. manshuriensis*) was discovered only by incident in the course of a screening for $GABA_A$ receptor modulators (Rueda et al., 2012). The commercially available sample of Bupleuri radix revealed to be a nearly 1:1 mixture of Bupleuri radix and the nephrotoxic and cancerogenic adulterant Aristolochiae manshuriensis radix. Bupleuri radix is a very expensive herbal substance. Therefore, it may even have been adulterated by intent (Körfers and Sun, 2008), which may have serious implications for health, if the adulterant is Aristolochiae manshuriensis radix. Due to the high risk potential of the consumption of AA containing herbs, a representative species out of

the Aristolochiaceae family, *Aristolochia clematitis*, was chosen exemplarily in this work (see 4.5), to elucidate the retraceability of this species with the established methods. AAs, produced by *Aristolochia clematitis* are known to be associated with the Balkan endemic nephropathy, a disease that has a high prevalence among people living in areas of the Balkans (Djukanović and Radovanović, 2003; Grollman et al., 2007).

1.3 The potential of complementary methods for the authentication of herbal substances

Despite the existence of established methods recommended for quality assessment of herbal medicines in regulatory guidelines, particular characteristics of finished herbal medicinal products like small amounts of samples, highly processed material or the discrimination between closely related plants require evaluation of alternative and complementary methods (Joshi et al., 2004; Ouedraogo et al., 2012; Sucher and Carles, 2008, see also: Figure 1). For morphological analysis, in-depth botanical knowledge is mandatory. Many medicinal plants, e.g. from European systems or TCM, are comminuted or even powdered into small pieces, hampering morphological identification. Special processing methods predominantly applied in TCM and ayurvedic medicine increase this problem (Zhao et al., 2006a).

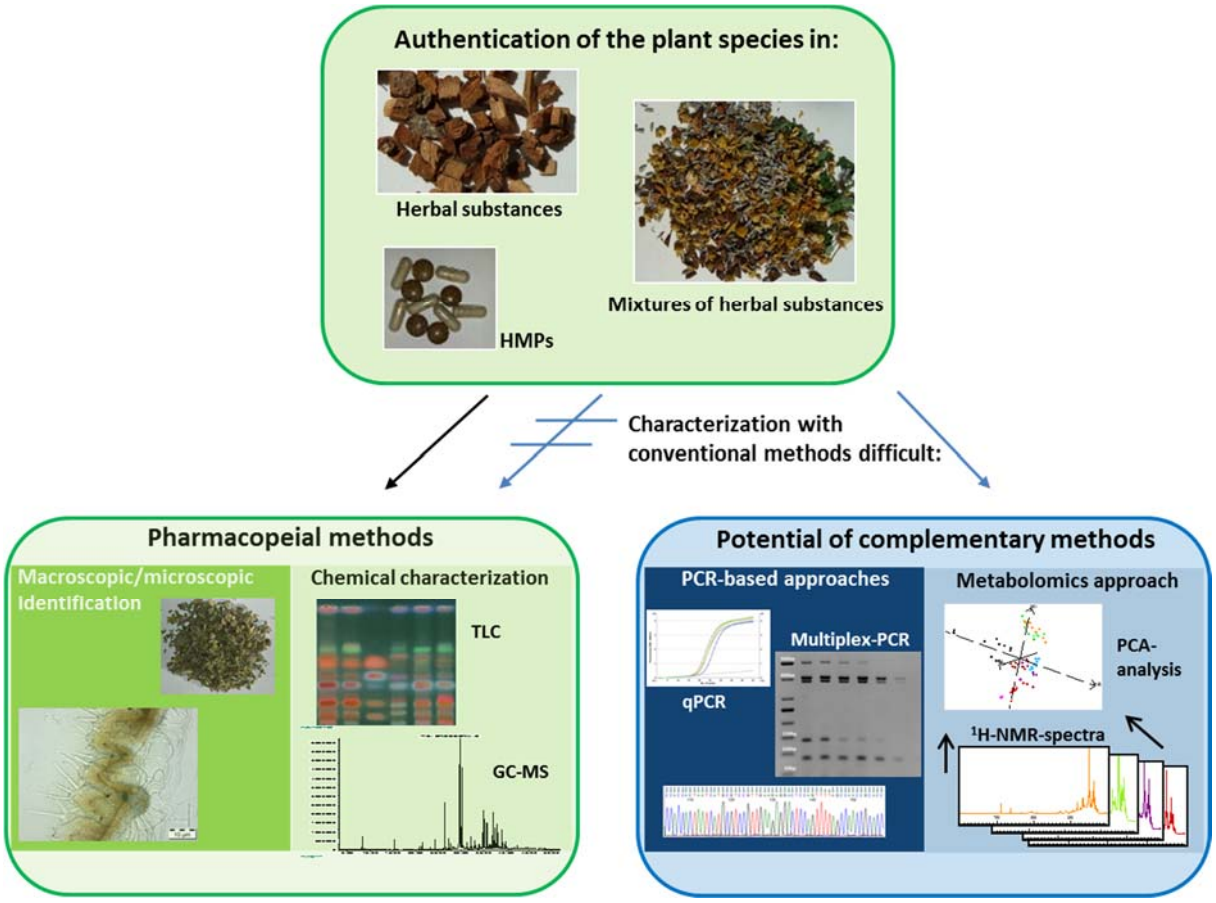


Figure 1: Schematic diagram: Pharmacopoeial methods for the authentication of herbal substances and the potential of complementary methods.

Chemical profiling for quality control is mainly based on marker substances (Razmovski-Naumovski et al., 2010). Unlike synthetic pharmaceutical compounds, herbal preparations comprise complex mixtures of components, belonging to different classes of natural compounds (Waszkuc, 2014). Furthermore, the chemical profile may vary from batch to batch depending on factors like growing area, time of harvest and climate. In many cases no active marker is known or available for a medicinal plant (Sahoo et al., 2010). Marker substances may as well be intentionally added to herbal substances to pretend the existence of the proper species (Zhang et al., 2012).

1.3.1 Pattern-oriented approach by metabolic fingerprinting of herbal substances

The metabolome is the final downstream product of the genome and represents the entirety of small molecular weight compounds existing in a cell or organism, participating in metabolic reactions (Fiehn, 2002). Due to a complex metabolic network,

required for vital functions of the plant like growth and maintenance, more than 200.000 metabolites are estimated to exist in the plant kingdom (Sumner et al., 2003). The term metabolics refers to the identification and quantification of all metabolites in a biological cell, tissue, organ or organism (Dunn et al., 2005). However to date, no analytical equipment is available to completely meet such a demand.

In general, metabolic fingerprinting is a metabolic approach, and implicates a comprehensive and high-throughput analysis of crude samples or sample extracts (Dunn et al., 2005). The effort for sample preparation is kept low. Thus, samples are directly analyzed without onward fractionation of metabolites into subclasses and identification or quantification is often not intended.

Among the methods used for fingerprint analyses, there are low resolution techniques like TLC or IR-based methods, which are mostly assessed by visual analysis of signal patterns, or high resolution techniques like GC, HPLC, NMR, MS or the hyphenation of techniques (Jiang et al., 2010). High resolution techniques thereby provide high sensitivity and an enormous set of data, which makes these techniques amenable for analyses of the metabolome. In this work, metabolic fingerprint analyses were based on data provided by ^{1}H NMR spectra of dichloromethane (DCM) extracts of dried plants. Advantages of ^{1}H NMR spectroscopy are a simple and fast sample preparation (Daniel, 2009) and the ability of sample classification on the basis of the metabolite profile without assigning individual peaks (Sheridan et al., 2012).

A clear disadvantage of NMR spectroscopy is the lower sensitivity in comparison to higher resolving techniques like mass spectrometry (Pan and Raftery, 2007). Nevertheless, ^{1}H NMR metabolic analysis is well established in the field of plant science and applied for quality control of herbal substances (Choi et al., 2004; Le Gall et al., 2004; Kim et al., 2005), detection of adulterants, retracing of species origin (Palama et al., 2011), for distinction of harvesting points (Kim et al., 2011; Palama et al., 2010), detection of genetic modifications in crop plants (Defernez et al., 2004) and even for differentiation of developmental stages (Abdel-Farid et al., 2007; Carrari et al., 2006; Lubbe et al., 2013).

Data gathered by ^{1}H NMR measurements are complex. An approach for making this comprehensive information accessible to statistical analysis, is the combination with multivariate data analysis, e.g. principal component analysis (Daniel, 2009).

1.3.1.1 Principal component analysis (PCA)

Metabolic analyses result in complex data from which the differentiating components need to be refined. Principal component analysis (PCA) in the field of multivariate data analysis is a tool to reduce the complexity of recorded raw data, thereby preserving most of the variation present in the data set (Jolliffe, 2002). The method was first described by Pearson in „On lines and planes of closest fit to systems of points in space“ as a geometric interpretation (Pearson, 1901), and then further developed to its current status by Hotelling (1933), who was name-giving (Svante Wold, 1987).

PCA describes data in a lower dimensional space of significant variables (Jolliffe, 2002; Ullah and Finch, 2013). The reduction of data is obtained by transforming the data to a new set of variables, or principal components. In a given matrix of data with n points, the first PC will be set along the direction of the largest variation, the second, orthogonal to the first, will represent the second largest variation of the second coordinate, and so on (Kessler, 2006). By these calculations, the essential data patterns can be separated from noise. Hence, i.e. from a set of different samples, outliers can be detected and classes can be delineated (Daniel, 2009).

1.3.2 The potential of DNA-based methods for the authentication of herbal substances

The authentication of plants based on DNA sequences has several advantages when compared to chemical analyses. The latter are prone to be affected by conditions of cultivation such as place and time of harvest (Kim et al., 2011; Lubbe et al., 2013) and environmental factors like photoperiod, climate and nutrient availability (Sahoo et al., 2010). Even different developmental stages may alterate the chemical profile of a plant significantly (Palama et al., 2011). Moreover, TCM or ayurvedic preparations typically contain a mixture of different plant species that result in a highly complex chemical profile, which is difficult to interpret.

These factors may hinder an unequivocal identification of the respective herbal substances. In plant sciences, recent research has proven that DNA-associated methods provide a powerful approach to complement chemical analyzes for authentication of medicinal plants (Heubl, 2010; Kersten et al., 2008; Zhao et al., 2006b). DNA is a rather stable long-term evaluable molecule, resistant to many

external factors and unique for each species. Very small amounts are sufficient for analyses. DNA is not tissue specific, hence, can be isolated from every kind of plant tissue and independent from developmental stages. In addition, DNA often is a more powerful tool for resolving variances on the species level between closely related species compared to chemical analyses (Sucher and Carles, 2008).

For the authentication of herbal materials at the DNA-level, several techniques are applied in terms of phylogenetic studies or for matters of quality control. These can be divided into non-PCR-based methods (e.g. restriction fragment length polymorphism (RFLP)), PCR-based methods, PCR-based methods with subsequent sequencing and DNA microarray technology (Heubl, 2010; Kersten et al., 2008, Orland, 2014).

Nevertheless, DNA-based methods for quality control cannot replace chemical approaches, due to several limitations. First of all, PCR-related methods are dependent on successful isolation of high quality DNA, which has shown to be a problem especially in case of processed herbal material (Shaw et al., 2002). Methods applied for processing, such as high temperature or pH-variations can lead to degradation of DNA. Despite these processing procedures, PCR amplification might still be possible, when a short sequence of a marker-region is chosen. Additionally, high concentrations of inhibitory secondary plant metabolites, like i.e. phenolics, polysaccharides, essential oils (Bashalkhanov and Rajora, 2008; Demeke and Jenkins, 2010; Heubl, 2010) may impede DNA isolation or subsequent PCR reactions.

1.3.2.1 Internal Transcribed Spacer (ITS) and External Transcribed Spacer (ETS) as molecular marker

Internal Transcribed Spacer (ITS)

The term DNA barcoding implicates the analyses of a standardized DNA region aiming at the fast and precise identification of the respective species. It was first time introduced in a publication dealing with barcoding of *Plasmodium falciparum* by Arnot et al in 1993. Later it was strongly promoted by Hebert et al. (2003a) and the Consortium for the Barcode of Life (CBOL), an international initiative established in 2004, dedicated to evolve DNA barcoding as a global standard for identifying species (www.barcodeoflife.org). In mammals, a sequence in the gene encoding the cytochrome c oxidase 1 sebnquence has proven to be widely applicable for barcoding

(Frézal and Leblois, 2008; Hebert et al., 2003b; Waugh, 2007), but due to highly differing nucleotide substitution rates among plant and animal mitochondrial DNAs (mtDNAs), this sequence is not useful for barcoding of plants. While, in mammals, the nucleotide substitution rate is five times faster in mitochondrial DNA than in nucleotide DNA, this is not true for plant mitochondrial DNA (Wolfe et al., 1987). Strikingly, in DNA of flowering plants, mtDNA evolving occurs five times decelerated compared to nuclear sequences (Wolfe et al., 1987). Based on preliminary findings of ITS length conservation in association with high ITS nucleotide sequence variability, the ITS region was considered to have a potential use for plant phylogenetic studies (Baldwin, 1992). Since then it evolved to be the most commonly used barcoding region in plant phylogenetic studies at the species level (Chase et al., 2005; Kress et al., 2005; Lahaye et al., 2008), possessing high interspecific divergence (Kress et al., 2005). It was also considered for quality control of medicinal plants (Chiou et al., 2007; Howard et al., 2009; Pang et al., 2013; Xue et al., 2007a; Zheng et al., 2009) and, in this field, was successfully applied for authentication of a broad range of herbal substances in three former PhD theses in our group (Daniel, 2009; Kersten 2013, Orland, 2014).

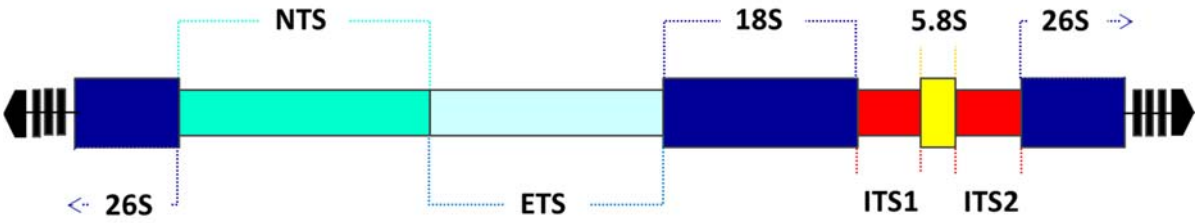


Figure 2: Schematic view of a part of the 18S–26S nrDNA repeat depicting the positions of the ITS1-, ITS2- and the ETS-region (Modified from Markos and Baldwin, 2001). NTS: Non-Transcribed Spacer, ETS: External Transcribed Spacer, ITS: Internal Transcribed Spacer

The ITS region is part of the transcriptional unit of nrDNA (figure 2), but the spacer segments ITS1 and ITS2 are not incorporated into mature ribosomes. They seem to play a role in the maturation process of nrRNA (van Nues et al., 1994). In angiosperms, ITS1 and ITS2 each possess a length less than 300 bp, ranging in size from 187 bp to 298 bp in case of ITS1 and 187 to 252 bp in case of ITS2 (for reported species, Baldwin et al., 1995). Hence, a separate amplification of ITS1 and ITS2, adjoining the 5.8S locus, is possible, which simplifies the amplification of processed and degraded DNA.

In some gymnosperms, the length of the ITS region varies between approximately 1.500-3.700 bp (Calonje et al., 2009).

The ITS region possesses several features rendering this sequence suitable for phylogenetic studies as well as authentication purposes. First of all, nrDNA is available in several dozens to thousands of copies in plants (Prokopowich et al., 2003; Simon et al., 2012), which promotes amplification by PCR. Additionally, the detection by PCR is simplified by the small size of the ITS region (<700 bp in Angiosperms) combined with the presence of highly conserved sequences flanking ITS1 and ITS2. Hence, the ITS region can be readily amplified using universal primers. In this project, we use specifically designed plant primers (Kersten, 2013). The most favorable feature of the ITS region is the fact that the nrDNA gene family has evolved strictly concertedly (Appels and Dvořák, 1982; Arnheim et al., 1980; Hillis and Dixon, 1991). This is achieved by gene conversion or unequal crossing-over. Variation is mostly attributable to point mutations (BG Baldwin et al., 1995).

The use of ITS for barcoding purposes also has limitations. It lacks some discriminatory power concerning species level variability in some taxa. Indeed, this problem especially accounts for phylogenetic studies, as the resolution provided by ITS is usually sufficient for authentication purposes in quality control of medicinal plants. Due to cases of divergent paralogues, cloning of multiple copies may be mandatory. Additionally, secondary structure problems may hinder PCR amplification. GC-rich sequences may be an obstacle for successful amplification (Henke et al., 1997; Sarkar et al., 1990), as was the case in this work for the ITS regions of *Salvia* species (4.1.3). Hence, an alternative region was additionally chosen for barcoding for these species, the External Transcribed Spacer (ETS).

External Transcribed Spacer (ETS)

The combinatorial use of at least two barcodes for plants was proposed several times in literature, e.g. the combination of ITS and a region in the chloroplastidal DNA, the trnH-psbA intergenic spacer, was suggested (Kress et al., 2005) to overcome limitations in phylogenetic studies. The simultaneous use of ITS- and ETS-sequence data for plant barcoding purposes was applied by Baldwin and Markos (1998), Bena et al. (1998) and Plovanich and Panero (2004), amongst others.

In eukaryotes, the 18S, 5.8S and 28S rRNA genes are arranged in tandem repeats separated by the two Internal Transcribed Spacers ITS1 and ITS2 and headed by an External Transcribed Spacer (ETS) (see Figure 2). The transcriptional units are adjoined by the Non-Transcribed Spacer region (NTS). The intergenic spacer (IGS), consisting of ETS and NTS, contains different regulatory elements, involved in transcription initiation and termination (McMullen et al., 1986; Rathgeber and Capesius, 1990; Rogers and Bendich, 1987; Volkov et al., 1996).

The ETS of flowering plants in general exceeds the length of the combined ITS1 and ITS2 region (Bena et al., 1998; Volkov et al., 1996; see Table 1) and, in contrast to ITS1 and ITS2, there is no length conservation.

Table 1: External transcribed spacer length (taken from: Bena et al., 1998)

Genus / Species	ETS length [bp]	Reference
Medicago	5300	Cluster et al. 1996
Brassica	754 [1]	Tremousaygue et al. 1992
Nicotiana sylvestris	1441	Volkov et al. unpublished [2]
Nicotiana tomentosformus	2172 [1]	Volkov et al. unpublished [2]
Oryza sativa	1227 / 1487 / 1735 [3]	Cordesse et al. 1993
Raphanus sativus	740	Delcasso-Tremousaygue et al. 1988
Vigna radiata	1155 / 1604 / 2153 / 2226 [3]	Schiebel et al. 1989
Zea mays	529 / 823	McMullen et al. 1986

According to calculations by Baldwin and Markos (1998), the ETS region accomplishes a 1.3 to 2.4 fold higher nucleotide substitution rate than ITS1 and ITS2 in the species reported in their work (*Calycadenia*, Asteraceae). In general, the ETS-region seems to evolve at least as fast as the ITS region at the sequence level.

1.4 Herbal substances and plant-derived materials investigated in this thesis

1.4.1 Herbal substances from the Lamiaceae plant family

The species of the Lamiaceae plant family are mostly herbs or shrubs. They represent a widespread family, comprising about 236 genera and about 7.200 species (Harley et al., 2004), the largest genus being *Salvia*, with more than 900 species. The family has an almost cosmopolitan distribution. Many species are aromatic and mainly due to the high content of essential oils in these species, several of them are used as medicinal plants (Raja, 2012). The volatile oils are mainly composed of monoterpenes,

[1] putative length given by the authors

[2] Unpublished data were taken from EMBL database

[3] putative length of ETS estimated from the conserved promoter motive (TATA(G)TA) flanking the RNA polymerase I transcription initiation site

sesquiterpenes and phenolic compounds (Wu et al., 2012). The monoterpenes can be subdivided into the acyclic monoterpenes (e.g. linalool, geraniol, citronellol), the monocyclic monoterpenes (e.g. thymol, menthol) and the bicyclic monoterpenes (e.g. camphor, thujone). Substances of the class of the sesquiterpenes are e.g. α-bisabolol or farnesol. Thymol and carvacrol are examples for aromatic compounds that are present in the essential oils of the species *Thymus vulgaris*. Other medicinally active substances in some species of the family Lamiaceae are the labiate tannins that have an adstringent effect. Because of their medicinal relevance and due to the high representance of species from the Lamiaceae family in the Ph. Eur., this family was selected in this work. Additionally, the consideration that the high content of essential oils was suspected to have impact on the efficiency of DNA isolation and following PCR reactions contributed to the selection of species from this plant family for testing alternative methods for authentication. The high amounts of polyphenols have the ability to bind to nucleic acids and may thereby impede DNA isolation (Pirttilä et al., 2001).

1.4.2 Herbal substances from Traditional Chinese Medicine (TCM)

TCM is a holistic medical approach for the prophylaxis and treatment of diseases firmly anchored in different Asian cultures since it has been developed in China more than 2000 years ago (Xu et al., 2013). TCM encompasses different medicinal practices, such as acupuncture, moxibustion, Chinese herbal medicine, or Chinese therapeutic massage (*Tui na*) (Englert, 2014). The therapy based upon Chinese herbal substances accounts for the major share of treatments in TCM. Drugs for TCM are of botanical (11,146 species), animal (1,581) or mineral (80) origin (Sheng-Ji, 2001). Nowadays, Chinese herbal medicines are gaining in popularity also in many western countries, e.g. in European countries and the US, thus influencing healthcare systems worldwide (Heubl, 2010; Yip et al., 2007). Furthermore, they are valuable sources for natural products research, e.g. the screening for new therapeutic drugs. The artemisinins produced by *Artemisia annua* L. were found to have an anti-malarial activity (Ho et al., 2014; Mueller et al., 2000).

In China, herbal substances are authenticated by trained TCM experts and prescribed by TCM practitioners with special expertise in this field. However, in many western countries, the knowledge of experts in this field is not available. Additionally, there is

no harmonized legislation for herbal substances derived from Chinese medicine and in many countries, herbal products are regulated as dietary supplements, thus circumventing rigorous controls for quality and safety (Bauer and Franz, 2010b). This is especially alarming, as many severe intoxication incidents arose from the misuse of herbs from TCM (see 1.2) (Chan, 2009; Krell and Stebbing, 2013; Singhuber et al., 2009; Vanherweghem et al., 1993). According to the Chinese Pharmacopoeia 2010, 83 substances derived from Chinese Materia Medica are officially recorded and defined as toxic and categorized according to their degree of toxicity (Liu et al., 2013). However, some potentially very dangerous species like e.g. *Aristolochia* species or *Polygonum multiflorum* (Dong et al., 2014; Liu et al., 2013) are not listed here. Many species from Chinese herbal medicine remain on the market and are still traditionally used in many countries. An example is the use of AA containing species from the family Aristolochiaceae, which are still used in China, Hongkong and Taiwan (Poon et al., 2007), notwithstanding that intake of AA has serious impairments on health (see also: 1.2).

Quality control of TCM herbal substances is especially challenging, as the majority of Chinese herbal medicines are administered as mixtures containing traditionally two to twelve diverse herbal substances (Sheridan et al., 2012). These highly complex mixtures contain a comprehensive spectrum of chemical compounds. A further aspect is, that e.g. the origin of a herb influences its chemical profile, but also special post-harvesting processing procedures (*pào zhì*, see also table 26) may alterate the composition of chemical compounds significantly (Wang et al., 2009). An example for regional varieties are e.g. traditional geographic growing regions (*dao di* medicinal materials) that aim to provide herbs of best quality (Yip et al., 2007). Hence, herbal substances derived from TCM were found a suitable model for the evaluation of metabolic fingerprinting in combination with PCA and, due to the impact of processing methods on the quality of DNA, also a good model for testing the limitations of the PCR based approaches applied in this work.

1.4.3 Cimicifugae racemosae rhizoma

Actaea racemosa L. (syn. *Cimicifuga racemosa* (L.) Nutt) is the plant species from which the herbal substance Cimicifugae racemosae rhizome is collected. *A. racemosa* is a perennial plant from the Ranunculaceae family, which is native to the Eastern United States (He et al., 2006) where it is growing in the temperate climates east of the Mississippi River. The commercial sales of Cimicifugae racemosae rhizoma are mostly provided with raw material of the plant growing in the wild in the Appalachian forests in the Eastern United States (Johnson and Fahey, 2012). In ancient times this plant was used as a remedy to alleviate pain during childbirth and menses but also for general ailments like malaise of kidney, rheumatism and malaria (He et al., 2006). Nowadays, the medicinal application of Cimicifugae racemosae rhizoma is focused on female complaints, most notably in association with ailments associated with menopausal and post-menopausal symptoms (Firenzuoli et al., 2011). In Europe, a monograph on Cimicifugae racemosae rhizoma was released in November 2010 by the HMPC at the European Medicines Agency (EMA). After evaluation of data from preclinical and clinical studies, the use of Cimicifugae racemosae rhizoma with an adequate labelling was found safe within the recommended indications. In Europe, there are several authorized medicinal products containing herbal preparations of *A. racemosa.*

Figure 3: Cimicifugae racemosae rhizoma

A. racemosa contains cycloartane triterpene glycosides, isoflavones, alkaloids and phenylpropanoids (He et al., 2006), among which most beneficial attributes are ascribed to the triterpenes (Watanabe et al., 2002; Wuttke et al., 2002). The presence of the estrogenic isoflavone formononetin is controversially discussed in literature, although more evidence seems to affirm an absence of this substance (He et al., 2006; Jiang et al., 2006; Kennelly et al., 2002; Panossian et al., 2004). However, the so far assumed activity of the extract of *A. racemosa* as phytoestrogen, a supposed therapeutic affect due to which it was used in hormone replacement therapy (HRT),

could not be readily confirmed. Recent findings suggest an antinociceptive activity of Cimicifugae racemosae rhizoma extracts (Johnson and Fahey, 2012).

Cimicifugae racemosae rhizoma was chosen in this work due to case reports on liver toxicity following the use of this herbal substance or herbal preparations thereof (Chow et al., 2008; Guzman et al., 2009; Joy et al., 2008). In July 2006 EMEA and HMPC released a report on 42 cases of suspected hepatotoxic effects in patients that had consumed preparations of Cimicifugae racemosae rhizoma, among them four cases with serious and reliable hepatotoxic reactions (Firenzuoli et al., 2011). Following this alert, several clinical trials towards the safety of this herbal substance were performed, but a potential liver damage could not be approved in association with the use of pure Cimicifugae racemosae rhizoma (Firenzuoli et al., 2011; Mazzanti et al., 2008; Nasr and Nafeh, 2009; Teschke et al., 2009). Additionally, most case reports were only poorly documented, without botanical or chemical analyzes of the potential hepatotoxic samples (Firenzuoli et al., 2011; Nasr and Nafeh, 2009). Hence, one hypothesis to explain this toxicity is based on a possible adulteration or contaminations with other species, e.g. with *C. dahurica* (TURCZ.) MAXIM*, C. foetida* L., and *C. heracleifolia* KOM. (He et al., 2006; Johnson and Fahey, 2012; Naser et al., 2011). Furthermore, reports on intentional adulterations of *A. racemosa* preparations (Jiang et al., 2011; Mahady et al., 2008) were recently published.

1.4.4 Propolis as model system for mixtures with unknown content

Propolis is a chemically complex, resinous bee product, collected from buds or exudates of plants. There is a great variance in physical appearance. Depending on the origin of the propolis sample, the color may be yellowish, red, green, brown or almost black. The texture varies from hard and friable to elastic and soft. The term propolis refers to its Greek origin and means *pro*, in defense of and *polis*, city (Ghisalberti,1979). By this, the primordial biological function of propolis is well described, as bees utilize it to protect their hives from environmental damage.

Figure 4: Collection of propolis samples at the apiary of the University Bonn.

They seal openings in their hives with propolis, thereby keeping the inner hive temperature at around 37°C, and impeding the entrance of predators (Salatino et al., 2005). Carcasses of killed intruders are embalmed with propolis to prevent a bacterial infection of the hive, pointing out the antimicrobial activitiy of the resinous substance. Along with antimicrobial properties, propolis provides further biological activities, among them antiseptic, antifungal, antioxidant, antiparasitic and antiviral activities, immunomodulatory and hepatoprotective effects (Banskota et al., 2001; Burdock, 1998; Marcucci, 1995; Sawaya et al., 2004). It is used in folkmedicine, in cosmetology and in food industry for health foods and nutrition supplements and claimed to improve human health and prevent diseases. In Germany there have also been authorized traditional medicinal products. It is used as complementary therapy to treat mucositis and is believed to promote re-epithelialization and therefore applied as treatment on erosions of the skin or wounds (Reddy et al., 2013). In mouthwashes and toothpastes it is described to prevent caries and to treat gingivitis and stomatitis (Gómez-Caravaca et al., 2006; Koo et al., 1999).

Propolis consists of resin, waxes, aromatic and essential oils, pollen and other organic matter, and the proportion of these chemical substances varies depending on the origin of the propolis sample (Dobrowolski et al., 1991; Marcucci, 1995). Beeswax is secreted from *Apis mellifera*, while resin and volatiles are plant derived and obtained by cutting of vegetative tissues or collection of plant secretions (Ghisalberti, 1979, Salatino et al., 2005). After collection of the lipophilic materials, bees mix it with salivary and enzymatic secretions (Gómez-Caravaca et al., 2006).

So far, more than 300 substances were detected in propolis (Castro, 2001). However, its chemical characterization is very challenging as its composition may be different depending on the region of its origin. For the temperate zones, like North America, Europe and the non-tropical regions of Asia, *Populus* species are the main propolis plant sources (Bankova et al., 2000). The major pharmacologically active substances are flavonoids like galangin, pinocembrin and pinobanksin (Banskota et al., 2001). In contrast, for Alecrim-derived or green propolis from Brazil, bees use alternative resin sources, mainly *Baccharis dracunculifolia* for the production of propolis (Salatino et al., 2005; Teixeira et al., 2005). Prenylated phenylpropanoids are highly abundant and characterizing constituents in this type of propolis (Salatino et al., 2005; Teixeira et al., 2005).

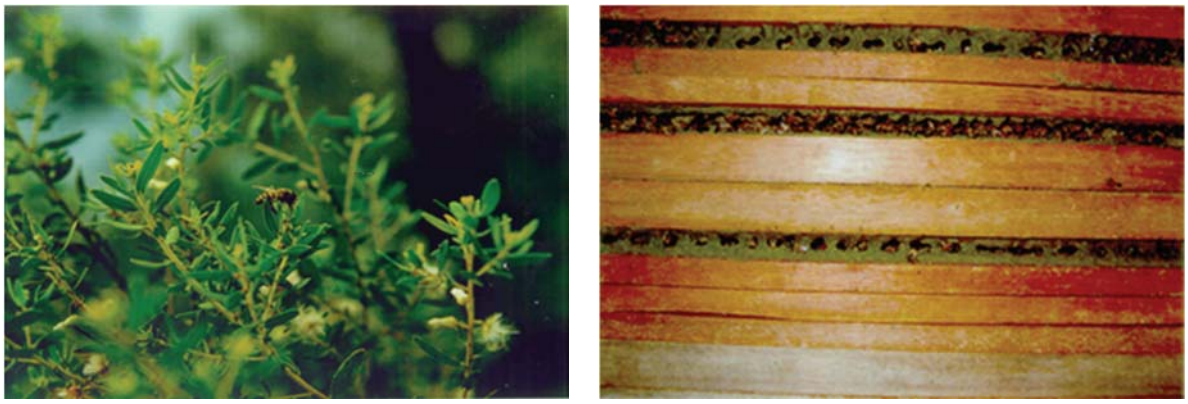


Figure 5: *Apis mellifera* collecting propolis of *Baccharis dracunculifolia* to production green propolis in Brazil (left side: *A.mellifera* cutting apices from *B. dracunculifolia*. right side: Deposition of green propolis in the hive. Taken from: Toreti et al., 2013)

As the pharmacological properties are assigned to plant derived substances in propolis, one focus of propolis research is its botanical source (Bankova et al., 2000; Salatino et al., 2005). In addition, the question was raised, whether knowledge of the plant source could be referred to as basis for a chemical standardization (Bankova et al., 2000). In the present work, the retraceability of plant sources of propolis was tested via PCR-related methods. Thereby propolis served as model for plant derived mixtures with unknown content, but it could be discussed, if this approach would also make sense for matters of quality control. The DNA-based investigations were very challenging, due to the sticky and resinous properties of propolis.

1.5 Investigation of defined mixtures of herbal substances (DMHS) based on various PCR-based approaches

Ineffective or even harmful plant adulterations can be found frequently in association with the application of plant medicinal products (Rueda et al., 2012; Vanherweghem et al., 1993). The impurities are either admixed or even substituted by intent (Joshi et al., 2004), for maximization of profits, or adulterated because of ignorance. There are guidelines on good agricultural and collection practice for starting materials of herbal origin (GACP), provided by the HMPC. Nevertheless, ambiguous declarations or nomenclature, or similarities in appearance of the herbal substances (Zhao et al., 2006a) may lead to misidentified herbal material. Additionally, in many cases there are only small amounts of plant material available, not sufficient for extensive analytical methods.

Hence, based on a PCR-based approach, already established in our working group (Kersten, 2013), further PCR-related methods were elaborated in this work, enabling the detection of plant components from mixtures of herbal substances, the composition of which is unknown. In addition, PCR-based methods were established and tested for the authentication of mixtures of herbal substances with defined composition using specific primers and probes.

1.5.1 Detection of different herbal components in DMHS with unknown composition by cloning

In this approach, the attempt was made to unravel unknown components in mixtures of herbal substances by universal primers amplifying the ITS region of DNA extracted from the plants. These primers are capable of annealing to the conserved regions of the 18 S and 26 S ribosomal DNA of a broad range of angiosperms. After isolation of DNA from the respective plant mixture, the ITS-regions were amplified by PCR with these universal ITS primers and the resulting DNA fragments were then ligated into a standard cloning vector (pGEM®-T, Promega). The formed plasmids were transformed into *E.coli*-cells and a defined number of colonies was selected and cultivated separately. Plasmid DNA was isolated and sequenced (see figure 6).

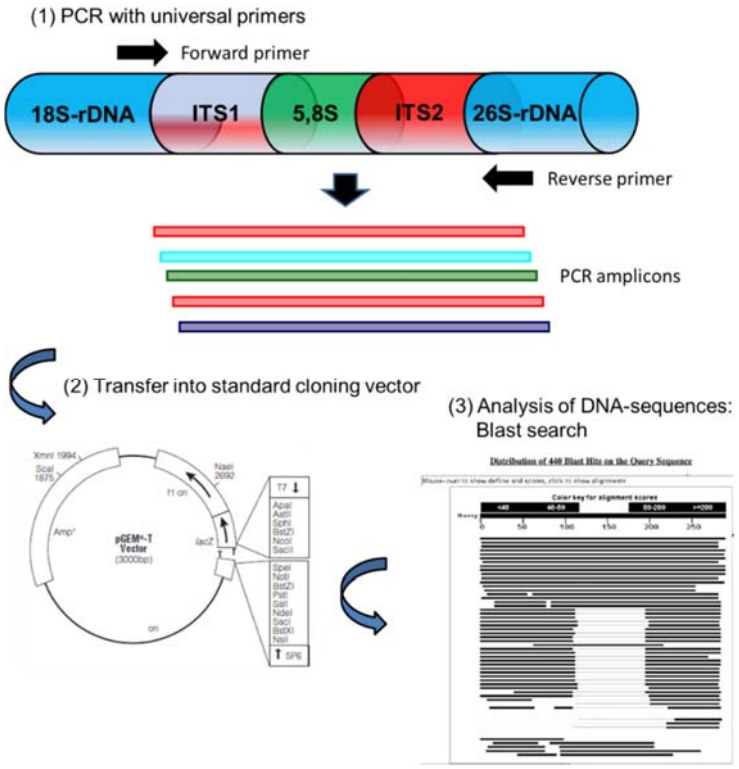


Figure 6: Approach for the identification of different components in mixtures of herbal substances by cloning. The ITS region of plant components is amplified with universal primers (1). The resulting amplicon is then transferred into a standard TA-cloning vector (pGEM®-T Vector System I, Promega, Mannheim) (2) and the successfully transformed cells were analyzed by isolation of plasmid DNA, subsequent check for positive transformants and following sequencing of the plasmid DNA of positive transformants. Finally, a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi, 2014) was accomplished (3).

1.5.2 Detection of different components in DMHS by multiplex PCR

As a method for the detection of different plant components in mixtures of herbal substances with known composition, a multiplex PCR for the parallel detection of five plant species was established (see figure 7). The term multiplex PCR generally refers to a PCR reaction that amplifies at least two target sites simultaneously and was first described by Chamberlain et al. in 1988. Due to the application of specific primers, this is a very sensitive approach, and favorable compared to single synthesis of multiple target sequences, (because of the parallel analysis of multiple samples). However, a multiplex PCR needs to be thoroughly evaluated. A major challenge in multiplex PCR

setups is the prevention of primer dimers and misprimed PCR products. They are preferably built at room temperature conditions during reaction setup and the first denaturation step. So called “hot start” conditions provided by a *Taq* polymerase, which is modified such that it needs an initial heating for 15 min at 95 °C for activation, leads to high PCR specificity. Additionally, the primers have to be designed carefully. Similar melting temperatures are needed for all primer pairs and at the same time, the PCR products have to differ sufficiently in length, to yield gel-resolvable amplicons. To overcome unspecific primer annealing, also a homology search should be carried out for each designed primer (BLAST). In terms of plant science, multiplex PCR-systems so far are particularly applied for the detection of genetically modified plant species (Xu et al., 2012), for the determination of nut allergens in food products (Hubalkova and Rencova, 2011), but also as alternative method for the quality control of medicinal plants (Chiou et al., 2007; Jigden et al., 2010; Xue et al., 2007b).

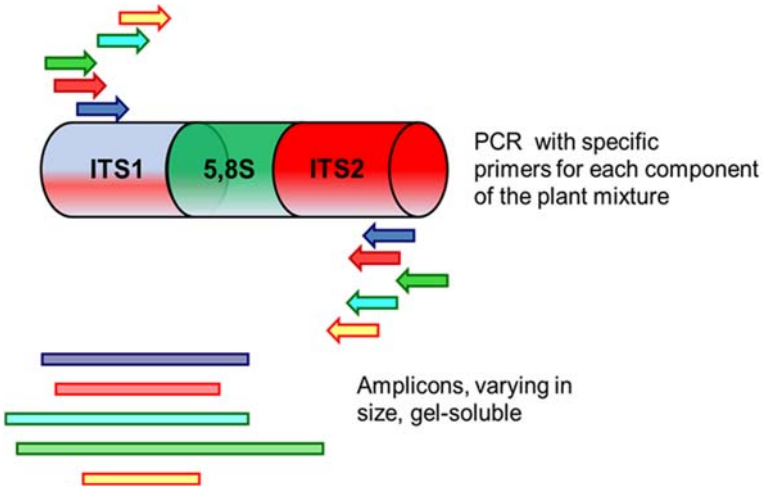


Figure 7: Approach for the detection of different components in mixtures of herbal substances by multiplex PCR. For each component of the mixture specific primers were designed based on the ITS region of the species. The primers were designed such, that the generated amplicons vary in size and are accessible for the identification by gel-analysis.

1.5.3 Detection of different components in DMHS with qPCR in combination with TaqMan® probes

A quantitative PCR (qPCR) assay was established in order to investigate defined mixtures of herbal substances. In comparison with the traditional endpoint PCR, qPCR

is a more susceptible approach, as the sensitivity is 100 to 1000 fold higher (Bustin, 2000; Mackay et al., 2002). Thus, the detection limit of an amplicon on an agarose gel is 2 to 4 ng, while for quantitative PCR conditions detection limits up to 2 to 20 pg can be achieved (Bustin, 2000; Mackay et al., 2002). The quantitative PCR enables a very precise determination of the initial quantity of target, as the amount of DNA is measured in the exponential range of the PCR reaction. The detection is achieved via fluorescent dyes, in this case TaqMan® probes, that yield an increasing fluorescent signal in direct proportion to the amount of PCR product molecules amplified (Heid et al., 1996; Livak et al., 1995).

The use of TaqMan® probes also increases the specificity of the PCR assay, as, in addition to the two primers, a third oligo-nucleotide, the probe, hybridizes with the DNA-template (Real Time PCR Handbook, 2012, Life Technologies). The TaqMan® assay is a so called 5´nuclease assay, which gained its name because of the 5´nuclease activity exhibited by the *Taq* DNA polymerase (Holland et al., 1991). Due to this activity, the *Taq* polymerase is able to degrade DNA bound to the template downstream of DNA synthesis. Furthermore, the TaqMan® assay is dependent on fluorescent resonance energy transfer (FRET) (Chen et al., 1997; Hiyoshi and Hosoi, 1994). FRET occurs, when the emission and excitation spectra of two fluorophores are overlapping and when the two fluorophores are in close proximity (Chen et al., 1997). This provides that the emissions of a fluorescent dye can be strongly reduced by the presence of another dye, the so called quencher. Before the PCR reaction, the reporter and the quencher have a natural affinity for each other, and the reporter signal is quenched. When the primers and the probe anneal to the target during PCR, the polymerase elongates the primer upstream of the probe and due to the 5´nuclease activity of the polymerase, cleavage of the probe takes place, releasing the reporter dye. The separated reporter dye is no longer quenched and its fluorescence can be detected (see figure 8).

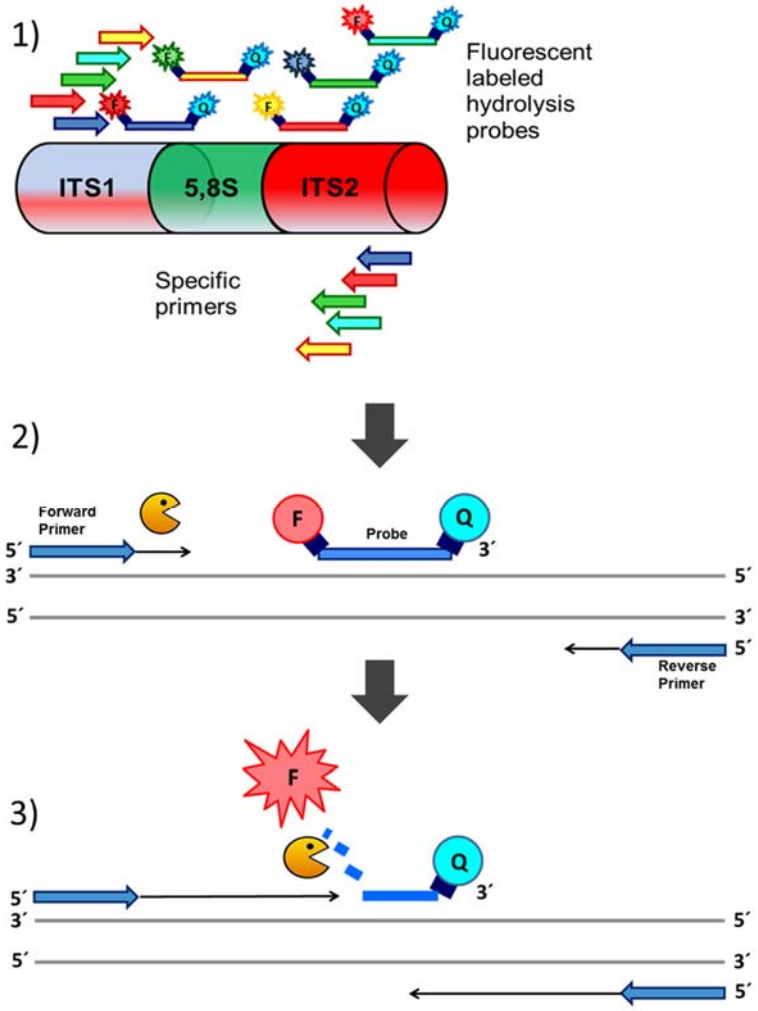


Figure 8: Approach for the detection of different components in mixtures of herbal substances by multiplex qPCR. 1) Specific primers and fluorescent probes were designed for each plant component (TIB MOLBIOL). 2) The *Taq* polymerase elongates the primers and with its 5´nuclease activity a cleavage (3) of the probe takes place. Now the fluorescence of the reporter dye is no longer inhibited by the presence of the quencher and the fluorescence can be detected. F - Fluorescent reporter dye; Q - Quencher dye

2 Scope of the study

The present work was prepared within the framework of a cooperation project of the Institute of Pharmaceutical Biology in Bonn with the Federal Institute for Drugs and Medical Devices. The central issue of this work was the evaluation and development of innovative methods for the unambiguous authentication of herbal substances and herbal preparations. An important requirement to ensure adequate quality of preparations made of herbal substances or mixtures is the authentication of the medicinal plants used therein. Indeed, appropriate methods for quality control are provided by the European Pharmacopeia, but particular challenges of finished herbal medicinal products like complex processing procedures and minor amounts of sample material, or the differentiation between close relatives within one genus have shown limitations concerning conventional methods. Here, methods were established that should serve as complementary methods for quality control supplementing already existing methods.

One core question being addressed in this work was the investigation of methods, capable of resolving the identity of different plant species in mixtures of herbal substances and finished herbal medicinal products. In this context, the potential of PCR-related methods, focussing on options provided by cloning strategies, multiplex PCR and qPCR was to be investigated. The cloning strategy thereby was to take into account a PCR-based method using universal primers targeting the ITS region of plants, already established and applied in former research projects (Daniel, 2009; Kersten, 2013; Orland 2014). In this work, the method was to be extended by means of a TA-overhang cloning step following the amplification of the PCR product, thus enabling the investigation of multiple different ITS amplicons in plant mixtures. Along with defined mixtures of herbal substances and finished medicinal products, propolis was to be investigated with this method as an example for a challenging matrix that is of unknown composition and contains a diversity of plant derived components. The multiplex PCR approach was envisaged to be based on the application of specific primers, to be able to concomitantly detect different herbal components in mixtures. For an even more specific and sensitive evaluation of plant mixtures, a qPCR method in combination with TaqMan® probes was investigated additionally to the latter method. For the establishment of these methods, herbal substances, finished herbal

medicinal products or plant-derived products referring to therapeutic systems of European and non-European origin were chosen.

An additional objective was the extension of an already existing database, providing metabolic analyses by ^{1}H-NMR-fingerprinting combined with multivariate data analysis (PCA) and an ITS-barcoding approach. Here, mostly herbal substances from TCM, made available by the EDQM, herbal substances of species from the Lamiaceae family, Cimicifugae racemosae rhizoma and propolis samples were to be measured by ^{1}H-NMR and evaluated with PCA.

3 Material and Methods

3.1 Materials

Chemicals were purchased in research grade or pro analysi quality. Ultra pure water was provided by a Milli-Q Water system and heat sterilized prior to use. It was used for the composition of media and buffers.

3.1.1 Devices

Table 2: Devices and technical equipment

Article	Manufacturer
Autoclave	Varioklav®, H+P Labortechnik AG (Oberschleißheim, Germany)
Biometra T3000 Thermocycler	Biometra GmbH (Goettingen, Germany)
Centrifuge Heraeus Biofuge *fresco*	Thermo Fisher Scientific (Waltham, USA)
Centrifuge Heraeus Contifuge Stratos	Thermo Fisher Scientific (Waltham, USA)
Eppendorf ThermoMixer®	Eppendorf AG (Hamburg, Deutschland)
Gel chamber Horizon 58, 11.14	Life technologies (Karlsruhe, Germany)
Incubator	Memmert GmbH + Co. KG (Schwalbach, Germany)
Inolab pH meter	WTW Wissenschaftlich-Technische Werkstätten GmbH (Weilheim, Germany)
Intas iX Imager	INTAS Science Imaging Instruments GmbH (Göttingen, Germany)
Laminar Airflow Clean Bench BSB 4A (Hera Safe, Class II)	Heraeus (Hanau, Germany)
LightCycler® 480	Roche GmbH (Basel, Switzerland)
Magnetic stirrer (IKA® RH basic)	IKA® Werke GmbH & Co. KG (Staufen, Germany)
Mill	Janke & Kunkel KG (Stauffen i. Breisgau, Germany)
Milli-Q® Water System	Millipore (Eschborn, Germany)
Multitron incubation shaker	Infors HAT (Bottmingen/Basel, Switzerland)
NanoDrop™ 1000 Spectrophotometer	Thermo Fisher Scientific (Waltham, USA)

Article	Manufacturer
Rotary evaporator, Laborata 4000 efficient	Heidolph Instruments GmbH & Co. KG (Schwabach, Germany)
Scale (Sartorius BL 3100)	Sartorius AG (Göttingen, Germany)
Scale (Sartorious Satorius BP 221S)	Sartorius AG (Göttingen, Germany)
TissueLyser	QIAGEN GmbH (Hilden, Germany)
Water bath (Haake DC 10)	Thermo Fisher Scientific (Waltham, USA)

3.1.2 Chemicals

Table 3: Chemical substances and solutions

Substance	Manufacturer
Aceton-d6 99,8% D Deutero	DEUTERO GMBH (Kastellaun, Germany)
Agar-Agar	Sigma-Aldrich Co. LLC (Switzerland)
Agarose	Agarose PeqLab (Germany)
Ampicillin	Roth Chemie GmbH (Karlsruhe, Germany)
Chloroform-d6 99,8%	DEUTERO GMBH (Kastellaun, Germany)
Boric acid	Roth Chemie GmbH (Karlsruhe, Germany)
Bromphenol blue	Roth Chemie GmbH (Karlsruhe, Germany)
Dichlormethan (HPLC Gradient Grade)	KMF LABORCHEMIE Handels GmbH (Lohmar, Germany)
DMSO	Roth Chemie GmbH (Karlsruhe, Germany)
dNTP-Mix	Promega GmbH (Mannheim, Germany)
Ethanol 99,8% p.a.	Roth Chemie GmbH (Karlsruhe, Germany)
Ethidium bromide	Roth Chemie GmbH (Karlsruhe, Germany)
Ethanol 99,8% p.a.	Roth Chemie GmbH (Karlsruhe, Germany)
Gel Loading Dye	Fermentas GmbH (St. Leon Rot, Germany)
Glycerol	Roth Chemie GmbH (Karlsruhe, Germany)
5× Green GoTaq®Flexi Puffer	Promega GmbH (Mannheim, Germany)
Isopropanol	Roth Chemie GmbH (Karlsruhe, Germany)
$MgCl_2$ x 6 H_2O	Merck KGaA (Darmstadt, Germany)
$MgSO_4$ x 7 H_2O	Merck KGaA (Darmstadt, Germany)
Na_2-EDTA	Roth Chemie GmbH (Karlsruhe, Germany)
NaCl	Merck KGaA (Darmstadt, Germany)

Substance	Manufacturer
NaOH	Merck KGaA (Darmstadt, Germany)
Na_2-EDTA	Roth Chemie GmbH (Karlsruhe, Germany)
SDS	Roth Chemie GmbH (Karlsruhe, Germany)
Sodium acetate	Merck KGaA (Darmstadt, Germany)
Tris	Roth Chemie GmbH (Karlsruhe, Germany)
Tris-HCl	Roth Chemie GmbH (Karlsruhe, Germany)
Tryptone/Peptone from Caseine	Roth Chemie GmbH (Karlsruhe, Germany)

3.1.3 Buffers and Media

Table 4: Composition of buffers and media used in this work

Buffer / Media	Composition
Buffer P1	50 mM Tris-HCl (pH 8), 10 mM EDTA, 100 µg/mL RNase A
Buffer P2	200 mM NaOH, 1% SDS
Buffer P3	3M potassium acetate (pH 5.5)
Tris-EDTA (TE)-buffer	10 mM Tris-HCl (pH 8.0), 1 mM EDTA
10x Tris-borate EDTA (TBE)- buffer	0.89 M Tris base, 0.02 M EDTA, 0.87 M H_3BO_3, H_2O ad 1 l
50x Tris-acetate EDTA (TAE)-buffer	242 g Tris 57,1 ml glacial acetic acid 100 ml 0,5 M EDTA pH 8,0 (1 M = 372,24 g/l) Ad 1 l Aqua dest., adjust to pH 8,3
Luria-Bertani (LB) medium	10 g tryptone, 5 g yeast extract, 10 g NaCl, H_2O ad 1 l, pH 7.5
LB-Agar	10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g Agar, H_2O ad 1 l, pH 7.5

3.1.4 Disposable material

Table 5: Disposable Material used in this work

Article	Manufacturer
Centrifuge tubes (15/50 ml)	TPP AG (Trasadingen, Germany)
Eppendorf tubes (0.5, 1.5, 2 ml)	Eppendorf AG (Hamburg, Germany)
Filter	Whatman GmbH (Dassel, Germany)

Article	Manufacturer
LightCycler® 480 Multiwell Plate 96	Roche GmbH (Mannheim, Germany)
Parafilm®	Pechiney Plastic Packaging Company (Chicago, USA)
Stainless Steel Beads, 5 mm	QIAGEN GmbH (Hilden, Germany)
Sterile filter (0.2 µm)	Renner GmbH (Darmstadt, Germany)

3.1.5 Kits, standards, vectors and enzymes

Table 6: Kits, standards, vectors and enzymes

Article	Manufacturer
Gene RulerTM 50 bp DNA ladder	Fermentas GmbH (St. Leon-Rot, Germany)
Gene RulerTM DNA Ladder Mix	Fermentas GmbH (St. Leon-Rot, Germany)
QIAquick PCR Purification Kit	QIAGEN GmbH ((Hilden, Germany)
QIAquick Gel Extraction Kit	Qiagen GmbH (Hilden, Germany)
DNeasy® Plant Mini Kit	Qiagen GmbH (Hilden, Germany)
Wizard® SV Gel and PCR Clean-Up	Promega GmbH (Mannheim, Germany)
PureYieldTM Plasmid Miniprep System	Promega GmbH (Mannheim, Germany)
DNA Clean & Concentrator TM 5	Zymo Research Corporation (Irvine, USA)
QIAGEN multiplex PCR Kit	Qiagen GmbH (Hilden, Germany)
LightCycler® Fast Start DNA Master HybProbe	Roche GmbH (Mannheim, Germany)
pGEM®-T Vector System I	Promega (Mannheim, Germany)
GoTaq® Flexi DNA Polymerase	Promega (Mannheim, Germany)
T4 DNA-Ligase	Fermentas GmbH (St. Leon-Rot, Germany)

3.1.6 Bacterial strains

Table 7: Bacterial strains

Strain	Genotype	Manufacturer
XL1-blue E. coli	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)]	Agilent Technologies GmbH (Böblingen, Germany)

3.1.7 Oligonucleotides

Primers and probes were provided as lyophilized powders by Eurofins Operon (Ebersberg, Germany) and TIB MOLBIOL (Berlin, Germany). For conventional PCR

applications, oligonucleotides were purchased in salt free quality, for real time PCR applications, HPSF (High Purity Salt Free) oligonucleotides were used.

Table 8: Universal primers used for ITS barcoding of herbal substances.

Primer	Sequence (5' - 3')	Reference
TKAfw	GAACCTGCGGAAGGATCATTG	Kersten, 2013
TKArev	GGACTACCCGCTGAGTTTAAGC	Kersten, 2013
TKBfw	GTTTCCGTAGGTGAACCTGC	Kersten, 2013
TKBrev	CGCGACCCCAGGTCA	Kersten, 2013

Table 9: Specific primers for amplifying a part of the ETS-region of *Salvia* species. Developed in this work.

Primer	Sequence (5' - 3')	Source
Salvia ETS fo 3	TTGGGCATGTTGGATCCCTG	This work
Salvia ETS re 392	GACTTGAACCGAGCTACGAG	This work

Table 10: Specific primers for propolis samples from "Propolis Elchingen" and propolis "Bonn". Developed in this work.

Species	Primer	Sequence (5' - 3')
Xanthium strumarum	Xanth str fo173	GGGCATCATGGATTTCACGTTG
	Xanth str re 632	GAGCATCTACACCCAAGAATGAAA
Trifolium repens	Trifol re fo251	TTTCGTGCGGGTTGTGTTCTGAC
	Trifol rep r568	CGTAACACAGCCACCACCTATCA
Trifolium pratense	Trifol pr fo 83	AGGGCTGGTTTGAGGTGTTC
	Trifol pr re552 j	GCTTAACACATGCACCATTTATC
Tilia platyphyllos	Tilia pla fo108	GAGCCCCTCCTCAGAGCCTGG
	Tilia pla re518	CCAGCAACGCCCTTAGCCTTT
Ambrosia artemisifolia	Ambrosia a fo127	TGTGAGGCCTTGTCGTTGTG
	Ambrosia a fo146	GTGTCTATGCTTGCACCATC
	Ambrosia a re 660	CAATGCGTCAGGGTACTTTA
Allium ursinum	Allium u fo128	AGAAGGAGAGCGGGAATAAG
	Allium u re 542	GGCTTGATGCGCACAATCTA
Helianthus annuus	Helianth a fo 66	TTGTCATGGATGTCATGTTG
	Helianth a re 583	CCAACACAAGACAGCCCTAT
Impatiens parviflora	Impatie pafo113	TCGGGTACAATTGCGTGTTC

Species	Primer	Sequence (5' - 3')
	Impatie pare 565	GGATACACCAAGGGTCAACA
Magnolia denundata	Magnol de fo146	CCCGGCTCCCTTTGAGGATG
	Magnol de re654	GGATGAGCTGAACGCCTGGT
Solidago virgaurea	Solida virfo61	CGGACGTCAGTTCGATTCTC
	Solida vir re548	CGCAACTGAATTCTGGGTTT
Solidago gigantea	Solida gigfo214	GTTGATGTGCGGCCTCGATG
	Solida gigre545	GGAAGGTTGGTGGGAGCGAC
Pinus sylvestris	Pinus sylvestris fo 94	GACACCTTTGTTTCTCTTTCTC
	Pinus sylvestris re 448	CAAGTTCGAGCGATGCCA
Brassica napus	Brassica napus fo 258	TGGTTGGATCGTACGCATAG
	Brassica napus re 638	CGGAACGTCTGACTATATGA
Fagus sylvatica	Fagus sylvatica fo 241	TCGACGTCTTGTATTTATCC
	Fagus sylvatica re 466	CTTCCGCCACCAAACGAGAT
Fagopyrum esculentum	Fagopyrum esculentum fo 188	AAGGACCACGAACAGAAG
	Fagopyrum esculentum re 542	CGTAGTCTTAGTACTCCACC
Castanea sativa	Castanea fo155	CCTCAACTCCGGTTCGGG
	Castanea rev262	GGGAACGAGGGTCGAAGAA

Table 11: Primer for multiplex PCR assays of plant mixtures and finished medicinal products. Developed in this work.

Species	Primer	Sequence (5' - 3')
Juglans regia	Jugl fo	AATGCCCCCTCCCAAAAAACG
	Jug rev	CCCACACATCATAAGAAGTGTTTG
Matricaria recutita	Matri fo	TGAGAAGGCTTGTTTCATGTTGCC
	Matri rev	ATCCTTGCGACCGACGACAC
Aristolochia clematitis	Aristo clem fo 88	ACGACACCCTGTGGTGACGG
	Aristo clem rev 238	CAAAGAGGCCTGGGAGCTTG
Quercus robur	Quercu fo	CGAATTGGTTACAACCGACG
	Quercu rev	CTCCGGCATGGCTCTC
Salvia miltiorrhiza	Salvi m. fo 568	ACAACTCACTTTCATGTCGTGATTC
	Salvi m. rev 633	GCACCGTGTGGCGCCTA

Table 12: Primer- and probe-sequences for the multiplex qPCR assay. (TibMolbiol)

Target	Name		Primer and probe sequence (5' - 3')
Juglans regia	J.regi S	F	GTGTGTGGTTGGTCAATCTTCTC
	J.regi A	R	GGTAAAGATGTCACCAACAACGC
	J.regi TM	Probe	FAM-CGCCCGTGGTTACTCCTTGTTT-BBQ
Matricaria recutita	M.recu S	F	GTCTGCCTGGGCGTCAC
	M.recu A	R	GTCCATCGAAGGGACTCCTATTT
	M.recu TM	Probe	610-CGCCATAAGCACGGGAGACCAATAT-BBQ
Aristolochia clematitis	A.clem S	F	GAACAGACACTCGGTGCAACC
	A.clem A	R	TCTCCATTGATGTGAGAGCCAAGA
	A.clem. TM	Probe	YAK-AGCGAAATGCAAAGCATGGACGACC-BBQ
Quercus robur	Q.robu S	F	CGGAAGGATCATTGTCGAAAC
	Q.robu A	R	CGGTTTGCTGGCAGGAGA
	Q.robu TM	Probe	640-TGCACAGCAGAACGACCCGC-BBQ
Salvia miltiorrhiza	S.milt S	F	GAACAACTCACTTTCATGTCGTGATT
	S.milt A	R	GCACCGTGTGGCGCCTA
	S.milt TM	Probe	670-TGCGTCGTCGGTATGGGCA-BBQ

3.1.8 Herbal substances and propolis samples

List of all samples investigated: see appendix

3.1.9 Software and databases

Basic **L**ocal **A**lignment **S**earch **T**ool (BLAST; http://blast.ncbi.nlm.nih.gov/) provided by the National Center for Biotechnology Information (NCBI) for alignments of nucleotide sequences.

ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) of the European Bioinformatics Institute (EBI) was applied for the generation of multiple alignments of nucleotide sequences.

The **CloneManager 9** software by Sci-Ed Software was used for the design of plant specific primers.

The sequence alignment editor **BioEdit** was used for alignments and manipulations of nucleotide sequence data (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

The **LightCycler® 480 Software**, release 1.5.0, provided by Roche GmbH, was applied for running real-time PCR applications. It was used for programming of PCR protocols, sample and template editing and different analysis modes for sample characterization.

Bruker **TOPSPIN 1.3 Software** was used for processing and calibration of ^{1}H-NMR spectra.

Bruker **BioSpin AMIX Software** was used for analyzing ^{1}H-NMR-spectral data with principal component analysis.

3.2 Molecular biological methods

3.2.1 Sterilisation

To avoid contaminations, all heatrestistant solutions, like buffers and media, as well as glassware and disposable materials were autoclaved for 20 min at 121°C and 2 bar atmospheric pressure in a Varioklav® steam sterilizer. Heat sensible solutions were sterilized by filtrating through membrane filters with a pore size of 0.22 µm.

Pure, deionised water was taken from a Millipore water purification system and autoclaved. Metal spatula, tweezers and scalpels were cleaned with water and a detergent, and then sterilized with ethanol.

3.2.2 Isolation of genomic DNA from plants and herbal materials

For the isolation of DNA, the dried plant samples were powdered in a TissueLyser (QIAGEN). Plant material was transfered into a 2mL Eppendorf tube and a QIAGEN stainless steal bead (5 mm diameter) was added. The mill was run for 2 minutes. If necessary, the procedure was repeated several times, until a fine powder was obtained.

Cimicifugae racemosae rhizoma: Dried roots and rhizomes were grinded with a mortar and pistil, then a small amount of about 50 mg per sample was filled into a reaction tube, one stainless steel bead was added and the sample was shredded again in a mill. For the DNA isolation, the powdered samples were processed according to the manual of the above listed DNA isolation kit (Invitek Food Kit).

Three different kits were used for the isolation of plant DNA. According to previous investigations by Kersten (2013), mainly the Invitek PSP Spin Stool DNA Kit was used as it proved to be the most effective kit especially for difficile samples like roots, rhizomes, cortices or processed plant samples. The QIAGEN plant mini kit and the Invitek Invisorb® Spin Food Kit II were used as well. All kits were used according to the instructions in the user's manual with small variations. As the PSP Spin Stool DNA Kit was originally designed for stool samples, instead of the recommended 200 mg sample material only about 20 to 50 mg of dried powdered plant material was used. If a high amount of plant material was applied, the solutions used in the course of the isolation were adapted to the higher volume. Also for the two other kits 20 to 50 mg of dried plant material were used for DNA extraction and if necessary, the amounts of solutions used were adapted. The DNA was eluted in elution buffer and then stored in a fridge at 4 °C or in a freezer at -20°C for long term preservation.

3.2.3 Cultivation of bacteria

Bacteria were handled using a laminar air flow clean bench to provide sterile conditions. For the cultivation of *E. coli-cells,* strain XL-1 blue (Stratagene, La Jolla, CA, USA) on agar plates, 50 to 350 µL of a liquid bacterial culture was plated on the agar using a disposable pipet-tip. Liquid cultures of single *E. coli* colonies grown on agar plates were cultivated in 1 mL LB medium in disposable 2 mL Eppendorf tubes. Subsequently, plates or liquid cultures were incubated at 37 °C in an incubator or shaker overnight.

3.2.4 Transformation of bacterial cells

Foreign DNA can be introduced into a bacterial cell via transformation of bacterial cells. Transformation by heat shock was applied. For this method chemically competent cells are needed.

3.2.4.1 Generation of chemically competent cells

For cloning procedures, *E. coli-cells,* strain XL-1 Blue (Stratagene, La Jolla, CA, USA) were used. To enable an integration of foreign DNA into these cells, they have to be modified chemically.

An over-night LB- pre-culture was inoculated with a single colony of XL-1Blue cells. This pre-culture was incubated at (37 °C) and constant shaking at 180 rpm. Then, respectively 1 mL of the pre-culture was transferred into 70 mL 2 x YT-medium, incubated at 18 °C and constant shaking at 180 rpm and grown to an OD of OD 600 of 0.3 - 0.4.

3.2.5 Ligation of DNA fragments for T-overhang cloning

PCR-products were purified and directly subjected to sequencing reaction, or ligated using the pGEM®-T Vector System I. This system provides linearized vectors with a single 3´-terminal thymidine at both ends for improving the efficiency of ligation. TA cloning benefits from the terminal transferase activity of some DNA polymerases such as *Taq* polymerase. The *Taq* polymerase adds a single, 3'-A overhang to each end of the PCR product, hence direct cloning into the linearized vectors with T-overhang is possible. Ligation takes place under the action of T4 DNA ligase. Due to the fact that the vector has a coding region for the enzyme β-galactosidase, also a blue/white screening is possible to detect vectors with inserted PCR products. The reaction mixtures were composed as follows:

Table 13: Composition of the ligation mixtures

Component pGEM®-T Vector System I	Volume/Quantity [µL]
pGEM®-T Vector	0.5 - 1
PCR amplicon	3 - 3.5
T4 DNA Ligase	1
2 x Rapid Ligation Buffer	5
Nuclease-free water	Ad 10

The ligation mixtures were then incubated for 1 h at room temperature or overnight at 4 °C.

3.2.6 Transformation of chemically competent cells

Transformation is a process that introduces exogenic DNA into a bacterial cell (competent *E. coli* XL1-blue cells), used for enrichment of vector DNA. An aliquot of

competent cells was thawed on ice and 10 µl of the ligation mixture was added. After incubation on ice for 1 h, the sample was heat-shocked for 90 sec at 42 °C in a water bath to enable the incorporation of DNA into the competent bacterial cells. Then, the cell suspension was again placed on ice for 2 min. To promote cell growth, 350 µl of sterile LB-medium was added and incubated at 37 °C for 45 min to 1h, while continuously shaking using the Thermomixer Eppendorf. An antibiotic selection was performed spreading 100-350 µl of this pre-culture on LB agar plates supplemented with the antibiotic amplicillin. The plates were incubated overnight at 37 °C using an incubator. After plasmid DNA isolation, the successful transformation of the vector construct was checked by PCR.

3.2.7 Isolation of plasmid DNA from bacteria

Following transformation and subsequent cultivation of transformed bacteria on agar plates containing ampicillin (100 µg/mL), (see 3.1.3), single colonies were grown in a 1 mL liquid culture over-night. Bacterial plasmids were gained by DNA isolation with the Promega PureYield Miniprep System, the Qiagen Plasmid Mini Kit or by a not column-based system. The kits were used according to the manufacturer´s instructions. When high amounts of colonies had to be screened for positive transformants, the alternative method without columns was used. Cells were pelleted by centrifugation at 8.000 rpm for 5 minutes, and, after removal of the supernatant, resuspended in 350 mL buffer P1. Subsequently, cells were lysed by adding buffer P2 to the cell suspension. After 5 minutes of lysis, the mixture was neutralized by adding 350 mL buffer P3.

Plasmids were purified from this culture using either the Promega PureYield Miniprep System or the Qiagen Plasmid Mini Kit following the manufacturer’s instructions. Alternatively, cells were pelleted by centrifugation at 13,000 rpm for 2 minutes and resuspended in 350 µl buffer P1. Cells were lysed by addition of 350 µl buffer P2 and the mixture neutralised by mixing it with 350 µl buffer P3. Cell debris and proteins were pelleted by 5 minutes centrifugation. Then the supernatant was mixed with 700 µL isopropyl alcohol and incubated at room temperature to precipitate DNA. The mixture was then centrifuged at 13,000 rpm for 30 minutes; the supernatant was removed and washed with 70 % ethanol and again centrifuged at 13,000 rpm for 10 minutes. The latter step was repeated and then the supernatant was again removed by pipetting and

decanting. The Eppendorf tubes were turned around for removal of residual ethanol. The completely dried pellet was then resuspended in 50 µL of sterile water. The plasmid DNA was then tested for positive insertion of the target DNA by PCR. If colonies were positive, DNA was further purified by the DNA Clean & Concentrator™-5-kit for sequencing purposes.

3.2.8 Polymerase chain reaction

Polymerase chain reaction (PCR) enables the exponential amplification of a defined DNA target sequence placed between two primer regions. The method was developed by Kary Mullis in 1980 and exploits the ability of DNA polymerase to synthesize a new strand of DNA which is complementary to the provided template strand. Mostly, the thermostable polymerase isolated from *Thermus aquaticus* (*Taq* polymerase), was used for the PCR reactions.

PCR reactions were assembled according to a PCR protocol optimized by Kersten (2013). The composition was as follows:

Table 14: Composition of the PCR reaction mixtures.

Components	Volume
5x GoTaq® Flexi Buffer	5 µL
$MgCl_2$ (25 mM)	1.5 µL
dNTPs (each 10 mM)	1.0 µL
Go*Taq* polymerase (5U/µL)	0.25 µL
Forward primer (20 µM)	2.5 µL
Reverse primer (20 µM)	2.5 µL
Template DNA	0.5 - 1 µL
RNase-free water	Ad 25 µL

The reaction was carried out in a thermocycler according to the following scheme:

Table 15 Thermal profile used for the PCR runs on the Biometra T3000 Thermocycler

Step	Temperature [°C]	Time [min]
1) Initial denaturation	95	5
2) Denaturation	95	
3) Annealing	50 - 65 (depending on primers used)	
4) Elongation	72	
5) Final Elongation	72	10
6) Cooling	4	hold

Steps 2 to 4 were repeated 25 - 35 times

Annealing temperatures were chosen about 2-5 °C lower than the the T_m-values provided by the manufacturer and the Clone Manager software.

3.2.8.1 Multiplex PCR

The QIAGEN® Multiplex PCR Kit was used for all multiplex PCR reactions. HotStarTaq® DNA Polymerase, a modified form of a recombinant 94 kDa DNA

polymerase, originally isolated from *Thermus aquaticus* (QIAGEN® Multiplex PCR Handbook) provided a “hot start” activity. Hence, the enzyme is inactive at low temperatures and needs an initial heating step (15 minutes at 95°C) to get into an active state, thereby providing high PCR specificity and and minimizing the formation of primer dimers or misprimed PCR products (Chou et al., 1992). Another prerequisite for specific multiplex PCR reactions is a careful primer design. Primers were designed with the primer design software Clone Manager, and designed such that similar annealing temperatures for all primers used as well as gel-resolvable PCR amplicons were obtained. Additionally, a homology search with BLAST was performed for primer construction.

Primers were prepared in a 10x mix, containing each primer in a concentration of 2 µM. At the beginning, equal concentrations of all primers were used. Then, the primer concentrations of more or less abundant expressed targets were adjusted such that all targets were amplified almost equally (see table 16). The template DNA was measured spectroscopically with a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). DNA was then diluted to adjust the amount of DNA to 100 ng DNA which was inserted to each multiplex PCR reaction (for the composition and the thermal profile see table 16 and table 17).

Table 16: Composition of the multiplex PCR reaction mixtures. (Primer mix:after establishing the PCR conditions: variable primer concentrations, depending on the yield of the different PCR amplicons)

Component	**Volume [µL] (25 µL)**	**Volume [µL] (12.5 µL)**
2x QIAGEN Multiplex PCR Master Mix	12.5	6.25
10x primer mix, (primer for: *Q. robur*: 400 nM, *M. recutita:* 50 nM, *J. regia* 100 nM, *S. miltiorrhiza* 200 nM, *A. clematitis* 400 nM)	2.5	1.25
Template DNA (100/50 ng per reaction)	variable	variable
RNase-free water	ad. 25	ad. 12.5

Table 17: Thermal profile used for the multiplex PCR runs on the Biometra T3000 Thermocycler

Step	Temperature [°C]	Time
1) Initial activation of "Hot Star" *Taq* Polymerase	94	15 min
2) Denaturation	94	30 sec
3) Annealing	63	90 sec
4) Elongation	72	90 sec
5) Final Elongation	72	10 min
6) Cooling	4	hold

Steps 2 to 4 were repeated 36 times

3.2.8.2 Multiplex qPCR with TaqMan® probes

All multiplex qPCR reactions were performed using a Light Cycler® 480 (SN 20340) provided with the Light Cycler® 480 Software release 1.5.0 SP1 at the Federal Institute for Drugs and Medical Devices (BfArM). The LightCycler® Fast Start DNA Master HybProbe kit (Roche GmbH) was used for all qPCR assays. The multiplex qPCR was established as a TaqMan® probe-based assay, in which a specific hydrolysation probe, labeled with a unique fluorescent dye, was designed for each target DNA.

The probes were twofold labeled, with a specific fluorescent dye as a 5´- modification and a dark quencher (BBQ – Blackberry Quencher) as a 3´- modification. This kind of quencher inhibits fluorescence in the area of 580 nm, which is especially important for multiplex assays. Fluorescent dyes were used according to table 18.

Table 18: Fluorescent dyes used for fluorescence-labeling of the probes and respective detection ranges used by the Light Cycler® 480 Software.

Designed for template DNA of:	Fluorescent dye	Range of fluorescence detection [nm]
Aristolochia clematitis	6FAM (Fluorescein)	483 - 533
Matricaria recutita	YAK (Yakima Yellow)	523 - 568
Quercus robur	LC 610 (Light Cycler Red 610)	558 - 610
Juglans regia	LC 640 (Light Cycler Red 640)	558 - 640
Salvia miltiorrhiza	LC 670 (Light Cycler Red 670)	615 - 568

The multiplex qPCR was established for plant mixtures composed of *Q. robur, J. regia, A. clematitis, M. recutita* and *S. miltiorrhiza* (table 18). The probes and respectively two specific primer pairs (for the sequences see table 12) for each component of the mixture were designed and synthesized by a synthesis lab (TIB Molbiol). Comprehensive optimization steps regarding efficiency of primers, $MgCl_2$ concentration, adjusting of probe concentrations of more or less abundant expressed targets and specificity testing were required for the multiplex qPCR assay establishment (establishment of the assay: see results & discussion 4.5.3.). At the beginning all probes were adjusted equally (0.5 µL = 0.25 µM each probe), after probe titration (see table 19).

The qPCR reactions were assembled as described in table 19:

Table 19: Composition used for the multiplex qPCR reactions. First, 0.5 µL probe were used of each probe; after probe titration different concentrations of each probe were used (see table).

Component	Volume [µL] (20 µL)	Final conc.
H_2O, PCR grade	ad. 20 µL	
$MgCl_2$ (stock solution 25 mM)	2.4	4 mM
PCR primer (10 pmol)	1 each	0.5 µM
TaqMan® probe (10 pmol):		
J.regi TM	0.375	0.1875 µM
S.milt TM	0.75	0.375 µM
A.clem. TM	0.5	0.25 µM
Q.robu TM	0.75	0.375 µM
M.recu TM	0.5	0.25 µM
LightCycler® Fast Start DNA Master HybProbe; 10x conc.	2	1 x
Template DNA (50-100 ng/reaction)	0.5 µL each	

The following PCR parameters were used for the qPCR reactions with the Light Cycler® 480 Software release 1.5.0 SP1 (table 20):

Table 20: Thermal profile used for the Light Cycler® 480

Analysis mode	Cycles	Segment	Target temperature [°C]	Hold time	Acquisition mode	Ramp-rate (°C/s)
			Pre-Incubation			
None	1		95 °C	10 min	None	4.4
			Amplification			
Quantification	45	Denaturation	95	10 sec	None	4.4
		Annealing	60	30 sec	Single	2.2
		Extension	72	20 sec	None	4.4
			Cooling			
None	1		40	30 sec	None	4.4

As several probes used exhibit overlapping fluorescence, a color compensation experiment had to be run. Color compensation is used to subtract the overlapping emission spectra from a reporter dye into improper channels outside of its appropriate and dominant channel (Wittwer et al., 2001, Table 21).

Table 21: Programm settings used for color compensation for the Light Cycler® 480

Analysis mode	Cycles	Segment	Target temperature [°C]	Hold time	Acquisition mode	Ramp-Rate (°C/s)
			Pre-Incubation			
None	1		95 °C	10 min	None	4.4
			Amplification			
Quantification	45	Denaturation	95	10 sec	None	4.4
		Annealing	60	30 sec	Single	2.2
		Extension	72	20 sec	None	4.4
			Color Compensation			
Color Compensation		Denaturation	95	30 sec	None	4.4
			40	30 sec	None	1.5
			85	-	Continuous	0.03
			Cooling			
None	1		40	30 sec	None	4.4

For the detection format settings of the Light Cycler® 480 Software see table 22.

Table 22: Detection Format settings Light Cycler® 480 Software release 1.5.0 SP1

Excitation Filter	Emission Filter	Name	Melting Factor	Quantification Factor	Max. Integration Time
483	533	483 - 533	2	1.5	1
523	568	523 - 568	1.2	5	2
558	610	558 - 610	1.2	5	2
558	640	558 - 640	1.2	5	2
615	670	615 - 670	1.2	5	2

3.2.9 Agarose gel electrophoresis

Agarose gel electrophoresis is a method for analyzing DNA or RNA-molecules and fragments thereof due to their size and charge. Agarose gels with a concentration of 1 - 3.5 % Agarose were used. For preparing the gels and for filling the gel chambers TBE- or TAE-buffer was used (see table 6). If the nucleis acids were reused and a DNA extraction from the gel was performed, TAE-buffer was used. For separation of the nucleis acids an electrical field was applied, by which the negatively charged molecules were moved through the gel-matrix. For size analysis a DNA ladder was loaded as reference sample. To visualize migration of nucleic acids during electrophoresis and for increasing density of samples, the DNA was mixed up with loading dye. PCR-reactions were directly loaded onto the gel, as the PCR-mix was provided with a loading dye. Gels were run at 60 - 120 V, and stained with ethidium bromide after the run. For staining, gels were incubated in an ethidium bromide bath for about 4 minutes. To avoid background staining, gels then were washed in a water bath for at least five minutes. Ethidium bomide is a DNA intercalating agent which is fluorescing in orange colour after exposure to UV-light at a wavelength of 336 nm. The fluorescence is intensified strongly when ethidium bromide is bound to DNA. Results were documented with the Intas iX Imager.

3.2.10 DNA extraction from agarose gels

For extraction of DNA fragments from agarose gels after separation by electrophoresis, the appropriate fragment was excised with a clean scalpel and subsequently eluted from the gel with the help of the Qiagen gel extraction kit following manufacturer's instructions. Purified DNA was solved in sterile ultrapure water.

3.2.11 Verification of nucleic acid concentration and purity

The concentration and purity of the DNA used in experiments was verified with a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific).

3.2.12 Sequencing of plasmids and PCR-fragments

Sequencing of DNA from the purified PCR-products was performed by GATC Biotech AG (Konstanz, Germany) using an ABI3730xl after the Sanger dideoxy method

(Sanger et al., 1977). For sequencing reactions, either PCR-specific primers or general primers (T7 or SP6) were used.

3.3 Analytical methods

3.3.1 Extraction of herbal substances and propolis

Herbal substances: For ^{1}H-NMR-measurements of herbal substances, plant material was powdered by using a mortar and pistil. Samples were prepared by weighing 300 mg of the plant powder in case of herbal substances from TCM provided by the EDQM (due to the limited availability of samples). Concerning all other herbal substances, 500 mg of powdered plant material was used for the extraction with DCM. After this, dichloromethane extracts were made according to a protocol established in a former research project (Daniel, 2009): The powdered sample material was extracted with respectively 10 mL DCM for 15 minutes under occasional shaking. Subsequently, DCM extracts were filtrated through folded filters (Schleicher/Schüller), and the resulting filtrates were evaporated under reduced pressure with a rotary evaporator. Concentrated samples were stored at -20 °C or directly investigated under the specific conditions described in 3.3.2. The extractions were performed by triple determination.

Propolis: Samples of propolis were extracted according to the extraction of herbal substances with small variations in sample preparation. Respective samples were prepared according to the properties of the samples. Crude propolis samples were milled very shortly, if possible. If necessary, powdered samples were grinded additionally using a mill, but in most cases, they were directly applied for the extraction with DCM. Crude propolis samples that were too soft for milling were grinded in a mortar adding a small amount of DCM. The extractions were performed by triple determination. Per sample, 300 mg propolis was used and partially pretreated.

3.3.2 ^{1}H-NMR-analysis

Dried plant extracts were resolved in 500 µL deuterated chloroform. ^{1}H-NMR spectra were recorded on a Bruker Avance 300 DPX operating at 300 MHz at a constant temperature of 25°C.

^{1}H-NMR spectra were processed using Bruker TOPSPIN 1.3 Amix-Software. Spectra were referenced to residual solvent signals with resonances at 7.26 for $CDCl_3$.

3.3.3 Principal component analysis (PCA)

The ^{1}H-NMR-spectra were imported into the Bruker BioSpin's AMIX Software, for evaluation by PCA. The following settings were adjusted for generation of the bucket table:

For ^{1}H-NMR-spectra: Spectroscopy: ^{1}D NMR; ^{1}D-NMR bucket method: simple rectangular buckets; Left border: 10.0 ppm or 8.0 ppm; Right border: 0 ppm; Bucket width: 0.05 ppm; Integration mode: sum of intensities; Scaling: scale to total intensity; Exclusions: DCM-extracts: 7.25-7.3 ppm ($CHCl_3$-signal)

The bucket table was created after selection of the spectra. After that, a PCA was calculated. The following settings were adjusted:

Basic PCA; scaling of bucket variables: no scaling; suppress small variances: min. variance level 5 %; set default confidence level: 99 %; select number of PCs by: min. explained variance; min. explained variance: 95 %

Score plots were chosen for the graphical depiction of the results from PCA. The lengths of the PC-axes represent the percentage share of the respective PC for explanation of the total variance of the original data.

3.3.4 Thin layer chromatography (TLC)

TLC was carried out using TLC aluminium sheets silica gel 60 F254 (Merck). Two different protocols were applied:

TLC of Salviae trilobae/officinalis folium (Pachaly, DC-Atlas, 3. Aufl.): The test solution was prepared by extracting 0.5 g of the powdered herbal substance with 5 mL methanol under occasional shaking. The filtrate served as test solution. As reference solutions, 20 µL borneole and bornylacetate, 25 µL cineole and 20 µL thujone were solved in 5 mL methanol. Chromatograms of plant extracts were prepared by applying 20 µL of solution to the TLC plate and using toluol/ethyl acetate as mobile phases under saturated conditions. The migration distance was about 12 cm. Detection was performed by spraying with anisaldehyde-H_2SO_4 (0.5 mL anisaldehyde are mixed with 10 mL glacial acetic acid , 85 mL methanol and 5 mL H_2SO_4) and heating of the TLC

plate at 105 °C for 5 minutes. The result of the TLC was evaluated by daylight and under UV_{365}.

TLC of Salviae trilobae/officinalis folium (European Pharmacopoeia 2007):

Salviae officinalis folium The test solution was prepared by extracting 0.5 g of the powdered herbal substance with 5 mL anhydrous ethanol under shaking. The filtrate served as test solution. As reference solutions, 25 µL cineole and 20 µL thujone were solved in 5 mL anhydrous ethanol. Chromatograms of plant extracts were prepared by applying 20 µL of solution to the TLC plate and using toluol/ethyl acetate (95:5 [V/V]) as mobile phases under saturated conditions.The migration distance was about 15 cm. The detection was carried out by spraying with phosphomolybdic acid in anhydrous ethanol and heating of the TLC plate at 105°C for 10 minutes. The result of the TLC was evaluated by daylight.

Salviae trilobae folium The TLC was prepared according to the protocol for Salviae officinalis folium (European Pharmacopoeia 2007), with the exception that 0.3 g of the powdered herbal substance was used for the preparation of the test solution.

3.3.5 Gaschromatography (GC)

A steam distillation was performed to obtain the essential oils of *Salvia* species. For this purpose, respectively 50 g of sample material was powdered with a mill and subjected to the steam distillation apparatus after adding 500 mL water. The herbal material was extracted for 1.5 h.

The essential oils were diluted with DCM (*Salvia officinalis* TD 047: 1:240; *Salvia triloba* TD 051: 1:720; *Salvia officinalis* IG 008: 1:240 [essential oil : DCM]) and measured by GC. The measurements were performed using a Chrompack CP-9003 apparatus. As reference solutions, cineole (1:1000), thujone (1:1000), borneole (1:50) and bornylacetate (1:50) were respectively solved in DCM (reference substance / DCM [V/V]). (The reference substances borneol and bornylacetate were solid substances [0.01 g was solved respectively in 5 mL DCM; then diluted 1:50 in DCM [V/V]]. The spectra were integrated with the supplied Maestro software. Manual peak integration was selected.

3.3.6 GC-MS analysis of propolis samples

GC-MS analysis performed in cooperation with Prof. Chinou, University of Athens

Extraction and sample preparation:

Propolis samples were extracted for 24 h with 70% ethanol (1:10, w/v) at room temperature. The extracts were evaporated to dryness in vacuo. About 5 mg of each residue was mixed with 50 µL of dry pyridine and 75 µL of BSTFA and heated at 80 °C for 20 min. Reference compounds were subjected to the same procedure for silylation as about 1 mg of the pure compound was mixed with 10 µL of dry pyridine and 15 µL of BSTFA. The silylated ethanolic extracts and reference compounds were analyzed by GC-MS.

GC-MS analysis

The GC-MS analysis was performed with a Hewlett-Packard gas chromatograph 5890 series II Plus linked to a Hewlett-Packard 5972 mass spectrometer system equiped with a 30 m long, 0.25 mm i.d., and 0.5 µm film thickness HP5-MS capillary column. The temperature was programmed from 100 to 300 °C at a rate of 5 °C/ min. Helium was used as a carrier gas at a flow rate of 0.7 mL/min. The split ratio was 1:20, the injector temperature 280 °C, the interface temperature 300 °C and the ionization voltage 70 eV. Hexane solution of n-alkanes was separated under the above conditions. Linear temperature programmed retention indices (LTPRI) were calculated.

3.3.7 Microscopic analyses of propolis samples

Microscopic analyses of propolis samples carried out by Dr. Sophia Karabournioti, University of Athens

Method of analysis (Graikou et al., 2011):

200 mg of propolis were dissolved and homogenized in a solution of ethylic alcohol, chloroform and acetone (1:1:1), the sediment obtained by centrifugation and separated is dissolved in 20 cm^3 of 10% KOH solution. It is then boiled for 2 minutes, centrifuged and separated for a second time and then treated using the alcohol. The sediment is centrifuged and separated for a second time. The compound is prepared on a slide.

3.3.8 Microscopic analyses of dried plant material

The dried plant leaves were cut in cross section with a sharp razor blade, then boiled up in trichloromethane, covered with a cover glass and then examined under the microscope.

4 Results & Discussion

Authentication of herbal substances by complementary methods

In two former research projects (Daniel, 2009; Kersten, 2013), methods for the characterization of herbal substances by metabolic- and ITS barcoding were established. To elaborate specific applications and also limitations provided by these two fingerprint methods, different species from the Lamiaceae family, herbal substances from TCM, the herbal substance Cimicifugae racemosae rhizoma and the plant derived bee product propolis were investigated within the framework of this study. Particular emphasis was placed on the analysis of mixtures of herbal substances. For this purpose, three PCR-based approaches were established and the efficiency of identification of herbal components in mixtures was examined based on defined plant mixtures and finished herbal medicinal products.

4.1 Case study 1: Authentication of herbal substances belonging to the Lamiaceae family

Herbal substances from the Lamiaceae family were differentiated at the genus level and at the level of species (*Salvia* sp.) using metabolic fingerprinting. The authentication of the different *Salvia* species was not successful for Salviae officinalis folium and Salviae trilobae folium, thus, further characterizing methods were applied for the latter two species (figure 9).

In addition, ITS barcoding was used for the evaluation of herbal substances from the Lamiaceae family.

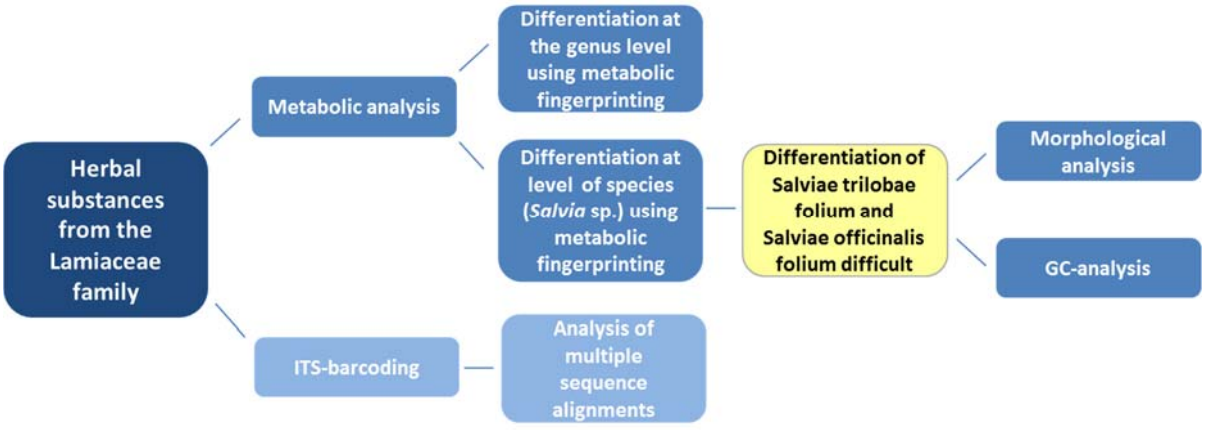


Figure 9: Schematic view of the strategy for analytical and PCR-based investigations carried out with herbal substances from the Lamiaceae family.

4.1.1 Differentiation of herbal substances from the Lamiaceae family at the genus level by metabolic fingerprinting

A set of species of the Lamiaceae family was selected to investigate the applicability of metabolic fingerprinting for differentiation of herbal substances belonging to the same family. It was evaluated, whether Lamiaceae can be distinguished with this approach at the genus level. Species of the genera *Salvia, Mentha, Rosmarinum*, *Marrubium* and *Thymus* were chosen.

Figure 10 shows the results of a PCA analysis of the selected genera. The same samples are shown in figure 11, but in this analysis, also the different species of the genera are displayed.

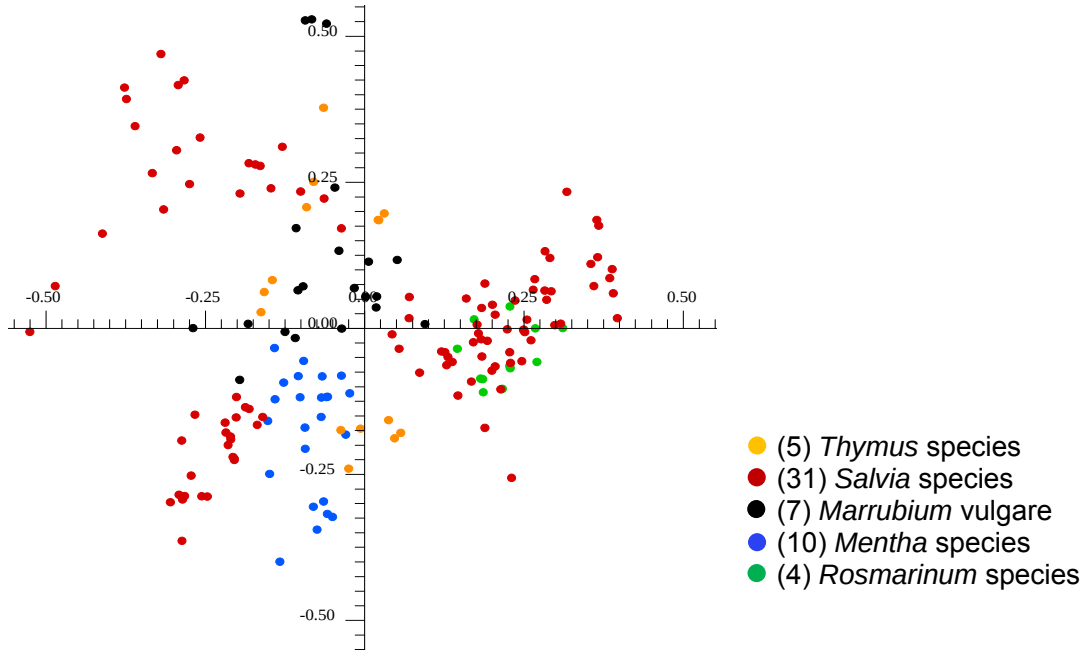


Figure 10: Score plot of PCA for PC1 and PC2 of ^{1}H-NMR-spectra of DCM-extracts of different genera from the Lamiaceae plant family. (Colouring according to the different genera. In brackets: number of samples investigated in triplicates).

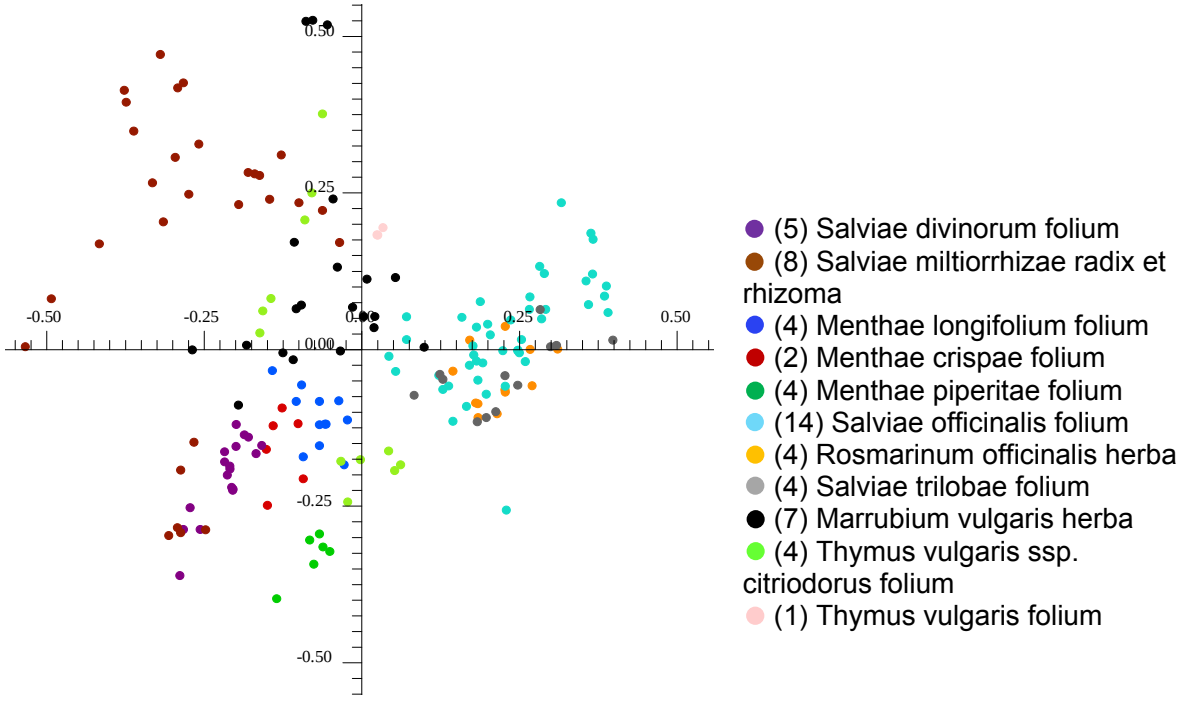


Figure 11: Score plot of PCA for PC1 and PC2 of ^{1}H-NMR-spectra of DCM-extracts of different species within the selected genera from the Lamiaceae plant family. (Colouring according to the different species. In brackets: number of samples investigated in triplicates).

Figure 10 demonstrates that different genera are not clearly discriminated by this PCA analysis. With respect to the genus *Salvia*, a discrimination into three different clusters is visible. Samples belonging to the genus *Thymus* are differentiated into two different clusters. A cluster is formed by samples of the three different species *Mentha longifolium* (Menthae longifolium folium), *Mentha spicata* (Menthae crispae folium) and *Mentha piperita* (Menthae piperitae folium) belonging to the genus *Mentha*. In this PCA it becomes apparent that due to similar substance classes present in different plant species, also different genera may overlap. This applies to the genus *Rosmarinum*, whose samples are overlapping with *Salvia triloba* (Salviae trilobae folium) and *Salvia officinalis* (Salviae officinalis folium) (figure 11). The essential oils of the Lamiaceae are likely to be extracted with the solvent dichloromethane, and Rosmarini folium, Salviae trilobae folium and Salviae officinalis folium shows similarities in terms of the composition of the essential oils, particularly regarding the three substances 1,8-cineole, borneol and camphor (see also table 23).

Table 23: Composition of essential oils of Salviae officinalis folium, Salviae trilobae folium and Rosmarini folium (Blumenthal et al., 2000; Wichtl, 2009).

Species	thujone [%]	1,8-cineole [%]	borneol [%]	camphor [%]	α-pinen [%]
S. officinalis	25-60	5-15	5-7	20-35	-
S. triloba	5-6	40-60	0.35	1.5-24	-
R. officinalis	-	15-55	3-6	10-25	10-25

This may be the reason for the lack of differentiation of Rosmarini folium, Salviae trilobae folium and Salviae officinalis folium. To evaluate, if a further classification of the three species is possible, they were subjected to another PCA analysis, in which only these species were compared (figure 12). In this PCA analysis, a differentiation of Salviae officinalis folium samples was achieved, while Salviae trilobae folium and Rosmarini folium still were not classified. Another PCA analysis including only Salviae trilobae folium and Rosmarini folium samples demonstrated that both species were not readily discriminated (figure 13). Figure 14 shows a PCA of Salviae officinalis folium and Rosmarini folium samples. The samples are discriminated with the exception of one sample of Rosmarini folium.

For a comparison of Salviae officinalis folium and Salviae trilobae folium samples see figure 16. Here, these samples were evaluated in the context of a more detailed analysis of samples belonging to the genus *Salvia* (4.1.2).

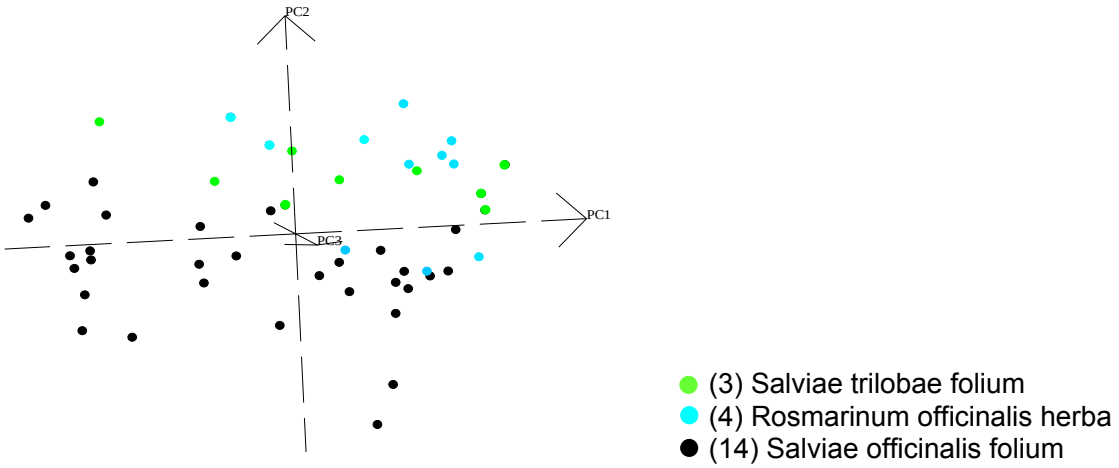


Figure 12: Score plot of PCA for PC1/PC2/PC3 of ^{1}H-NMR-spectra recorded from DCM extracts of Salviae trilobae folium, Salviae officinalis folium and Rosmarinum officinalis herba. (In brackets: number of samples investigated in triplicates)

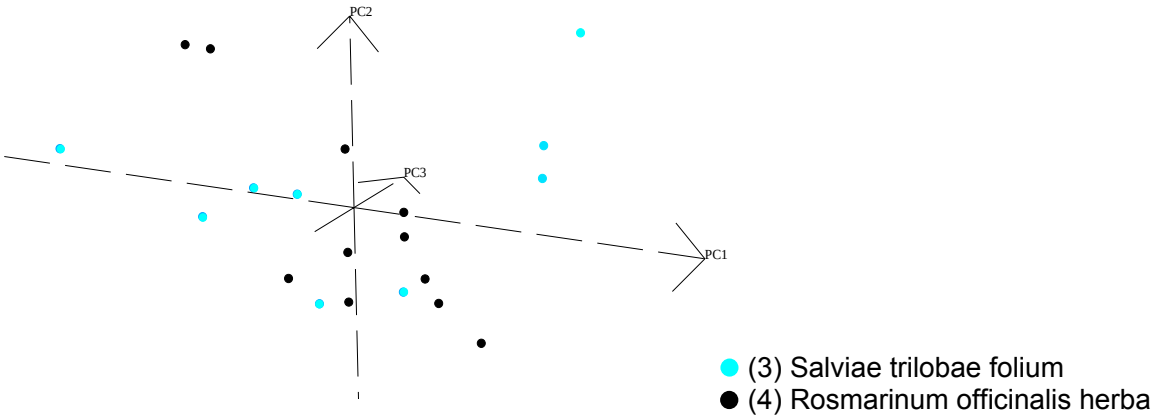


Figure 13: Score plot of PCA for PC1/PC2/PC3 of ^{1}H-NMR-spectra recorded from DCM extracts of Salviae trilobae folium and Rosmarinum officinalis herba. (In brackets: number of samples investigated in triplicates)

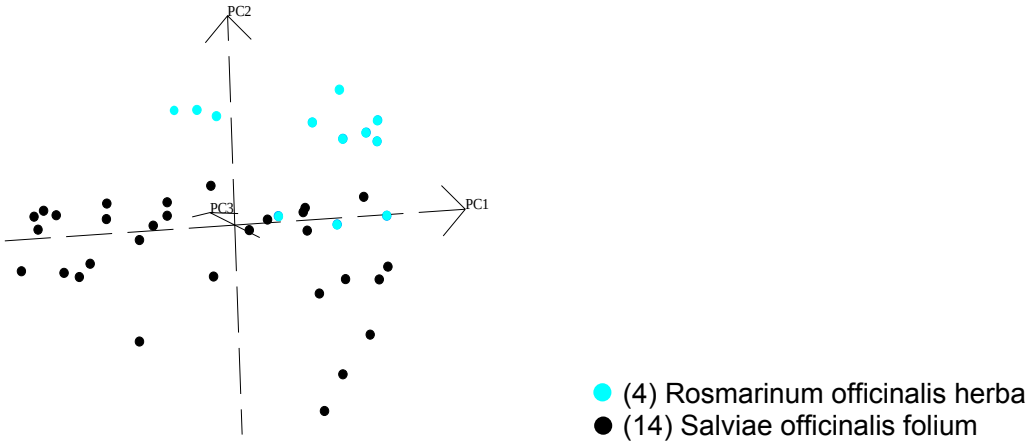


Figure 14: Score plot of PCA for PC1/PC2/PC3 of ^{1}H-NMR-spectra recorded from DCM extracts of *S. officinalis* and *R. officinalis*. (In brackets: number of samples investigated in triplicates)

The comparison of plants belonging to different plant genera shows that clustering is possible, when the compounds of the investigated plants of one genus are very specific for this genus and also consistent between different species of one genus (e.g. the investigated *Mentha* species, figure 11). Due to closely related constituents with similar basic structures that may occur in differing genera or families, also completely divergent plant species could cluster.

The broadest spectrum of different species was available for the genus *Salvia*, hence, more detailed investigations at the level of species in 4.1.2 were based on species belonging to the genus *Salvia*.

4.1.2 Differentiation at the level of species using the genus *Salvia* by metabolic fingerprinting

In this analysis, samples of wholesalers, pharmacies and the botanical gardens of the Pharmaceutical Biology were included, to cover a broad range of different plant samples. The options of distinguishing herbal substances by metabolic fingerprinting at the level of species was tested with the genus *Salvia*. Samples from five different species *S. officinalis*, *S. triloba*, *S. divinorum*, *S. milthiorrhiza and S. splendens* were subjected to PCA analysis. Samples from all species formed distinct clusters besides the two species S. *officinalis* and S. *triloba*, (figure 15). Accordingly, a discrimination up to the species level was observed for *S. splendens*, *S. divinorum* and *S. miltiorrhiza*.

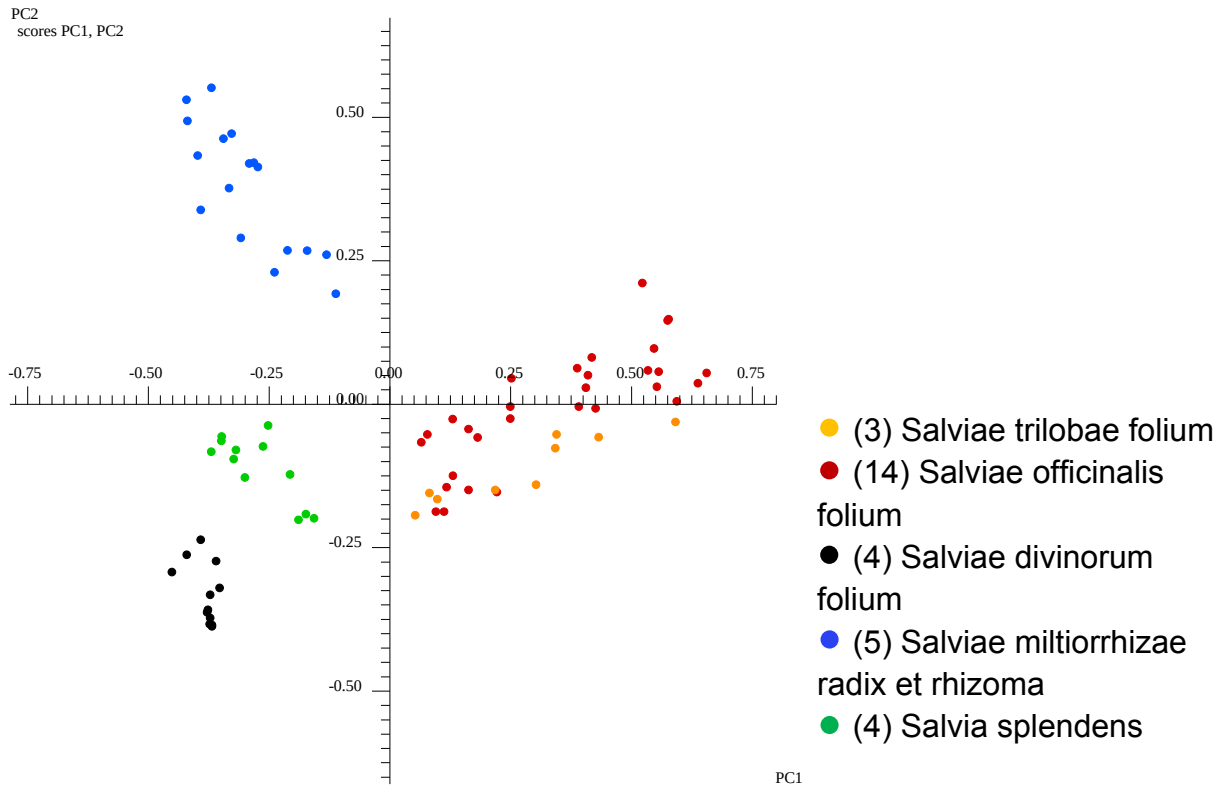


Figure 15: Score plot of PCA for PC1/PC2/PC3 of ^{1}H-NMR-spectra recorded from DCM extracts of different species from the genus *Salvia*. (In brackets: no. of samples investigated; all samples investigated in triplicates)

Figure 15 shows that all species were discriminated into different clusters with the exception of the two species *S. officinalis* and *S. triloba.* The latter two species were classified into one cluster by this PCA analysis of the ^{1}H-NMR-spectra.

For this reason, a second PCA was calculated, comparing only these two species (figure 16). In this analysis, reasonable classification of *S. officinalis* and *S. triloba* samples was obtained by PCA. However, *S. triloba* from the botanical gardens of the Pharmaceutical Biology (GPB) (Salviae trilobae folium IG 002) seemed to refer more to the group of *S. officinalis* than to the group of *S. triloba* (see red circle, figure 16). Therefore, it was supposed that Salviae trilobae folium IG 002 may be a hybrid of *S. officinalis,* hence, further investigations were carried out exemplarily.

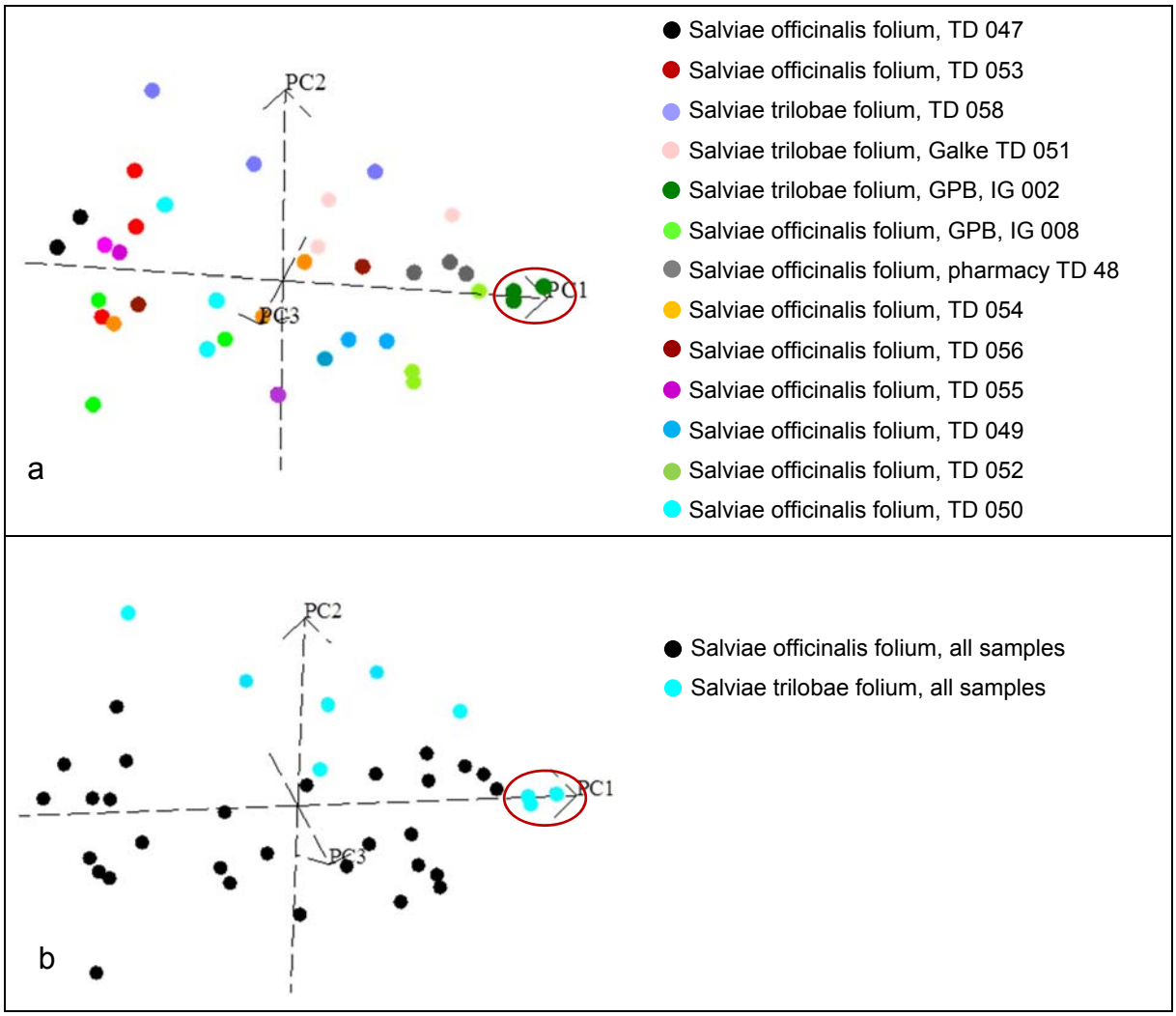


Figure 16: Score plot of PCA for PC1/PC2/PC3 of the ^{1}H-NMR-spectra of DCM extracts of Salviae officinalis folium samples and Salviae trilobae folium samples. a) all samples; colouring according to single samples. b) all samples; colouring according to species. All samples investigated in triplicates. (Red circle: Salviae trilobae folium, GPB, IG 002); GPB: botanical gardens of the Pharmaceutical Biology

One-dimensional loadings for PC1 and PC2 were investigated (see figure 17) for a more detailed description of the results shown in figure 16. The eigenvalues of the loadings correlate with the share of one original variable of the total variance. Higher eigenvalues correlate with a higher share of the variable of the total variance, hence they are more important for describing the original data than lower values (Kessler, 2006). The highest signals can be found in the upfield region (between 0 to 2.2 ppm), which is typically showing resonances of the protons of terpenoids, steroids and organic acids. Especially terpenoids, representing a major group of constituents of essential oils, may contribute to the total variance in the investigated *Salvia* species.

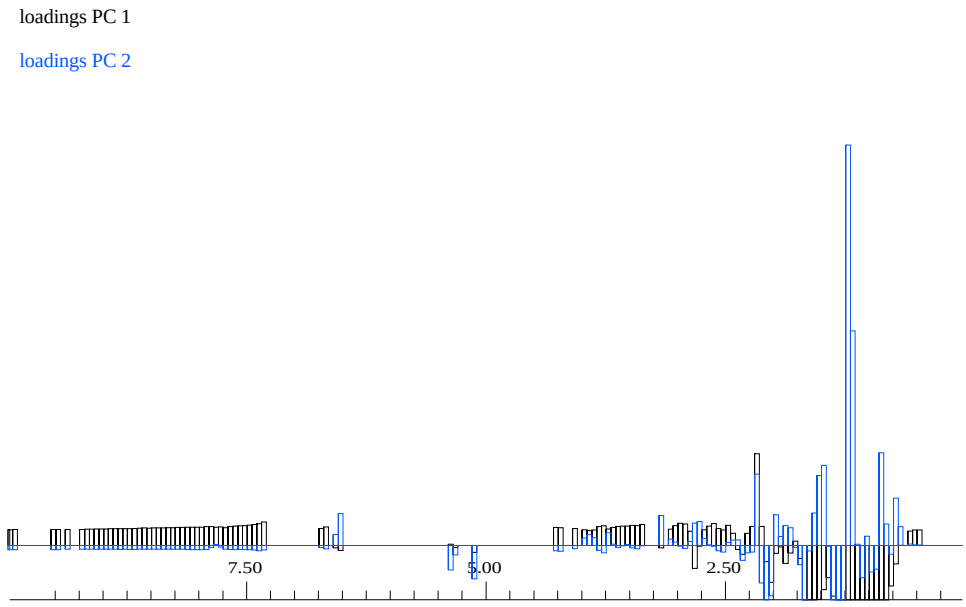


Figure 17: One-dimension loadings for PC1 (blue) and PC2 (black). The scaling corresponds to the chemical shifts [ppm] of the ^{1}H-NMR spectra.

Regarding the score plot of the PCA analysis (figure 16) and the loading plot it may be suggested that mainly constituents of essential oils are responsible for discrimination. Constituents of the essential oils are a major group of secondary metabolites in the Lamiaceae family (Agostini et al., 2009; Baratta et al., 1998; Bozin et al., 2007).

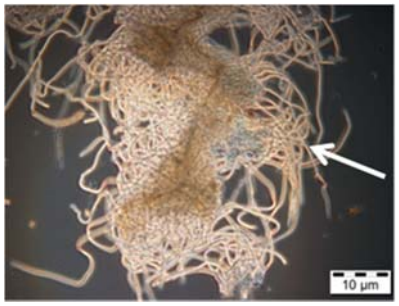
4.1.2.1 Morphological analysis of Salvia samples

For a further characterization of the different species of the genus *Salvia* according to methods described in the Ph. Eur. (the microscopic description), also a microscopic analysis was made for samples of *S. triloba* and *S. officinalis* (3.3.8). A characteristic morphological attribute for the differentiation between the two species are the trichomes of the plants. They are located on both sides of the leaves and in case of *S. officinalis*, both sides of the plants leaves are covered with multicellular, tortuous trichomes on both leaf surfaces (Wichtl, 2009). Distinct from *S. officinalis*, the leaves of *S. triloba* are not identical on both leaf surfaces. The hairs of the upper surface are stiff and upright. Additionally, leaves of *S. triloba* have a much denser tomentum compared to those of *S. officinalis* (Wichtl, 2009).

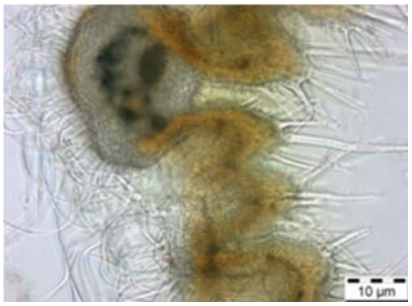
As reference samples, two certified samples also analyzed by GC-analysis (see: 4.1.2.2: Salviae officinalis folium TD 047 and Salviae trilobae folium TD 051), were included in this analysis. Two additional reference samples were investigated; the

samples Salviae trilobae folium IG 020 (gardens of useful plants University Bonn (GUP), identified by a botanist) and Salviae trilobae folium TD 058 (PhytoLab). The samples Salviae officinalis folium, GPB, IG 008 (also investigated in a GC- analysis; see 4.1.2.2) and Salviae trilobae folium, GPB, IG 002 were investigated, as their identity so far was not clear after PCA analyses (figure 16).

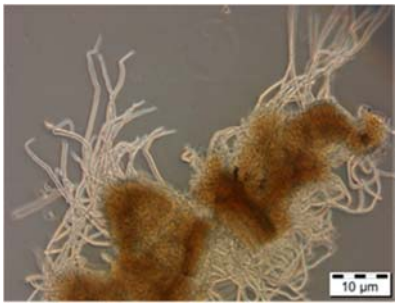
The pictures displayed in figure 18 confirmed the characteristics described above for most of the analyzed samples. However, the identity of Salviae officinalis folium, IG 008 and Salviae trilobae folium, IG 002 from the GPB was not clearly revealed with this microscopic analysis. Hence, further characterizing analyses were performed, using GC-analysis of the essential oils (4.1.2.2) and DNA-based analyses (4.1.3).



Salviae officinalis folium, GPB, IG 008, 1

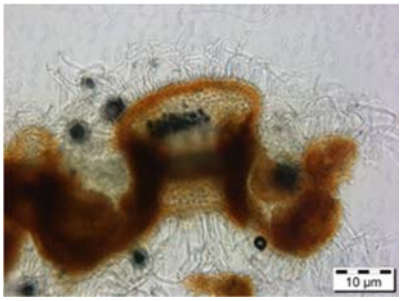


Salviae officinalis folium, GPB, IG 008, 2

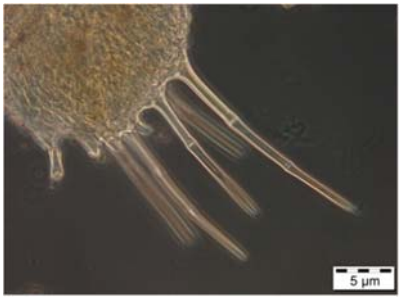


Salviae officinalis folium, TD 047

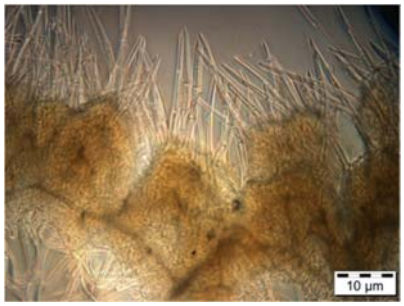
Salviae trilobae folium, Galke, TD 051



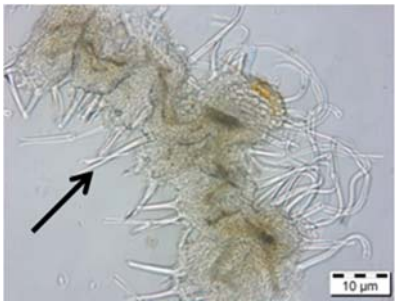
Salviae trilobae folium, GPB, IG 002



Salviae trilobae folium, GUP 1, IG 020



Salviae trilobae folium, GUP 2, IG 020



Salviae trilobae folium, TD 058 1

Salviae trilobae folium, TD 058 2

Figure 18: Microscopical analysis of Salviae officinalis folium- and Salviae trilobae folium samples. The dried plant leaves were cut in cross section with a sharp razor blade, then boiled up in trichloromethane. (white arrow: long, curly whiphair; black arrow: stiffened, straight hair); GUP: gardens of useful plants University Bonn; GPB: botanical gardens of the Pharmaceutical Biology

4.1.2.2 GC-analysis of essential oils from samples of Salviae officinalis folium and Salviae trilobae folium

Essential oils of dried plant samples were obtained by steam distillation (see material & methods: 3.3.5). Identification and quantification of the major constituents borneol, bornyl acetate, 1,8-cineole and thujone was performed by GC-analysis (see figure 19).

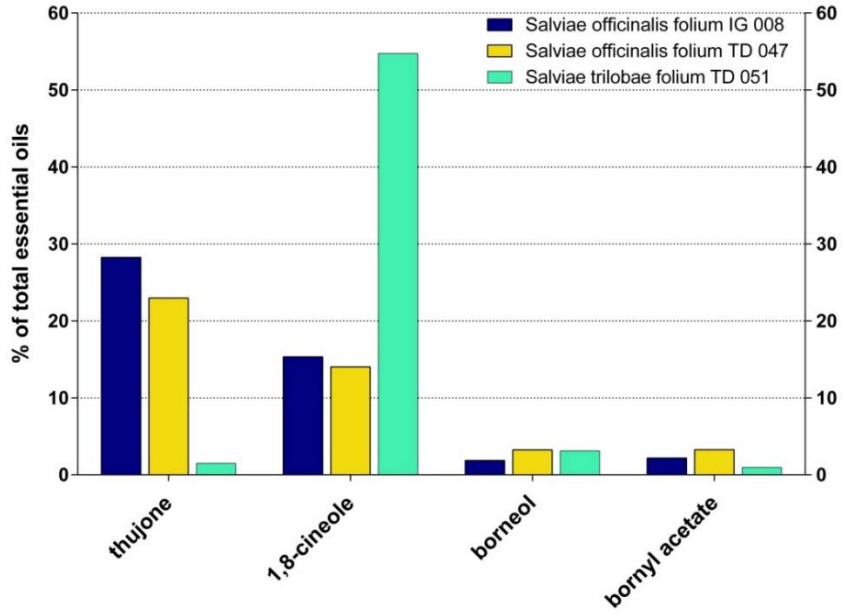


Figure 19: GC-analysis of the essential oils of Salviae officinalis folium and Salviae trilobae folium samples.

Three samples were analyzed, *S. officinalis* from the GPB (Salviae officinalis folium IG 008), *S. officinalis* from a pharmacy (Salviae officinalis folium TD 047) and *S. triloba* from the wholesaler Galke (Salviae trilobae folium TD 051). Only a limited set of *S. triloba*- and *S. officinalis*-samples was available for the GC-analysis and was analyzed exemplarily.

The GC-analysis of the different essential oils showed a composition that is typical for essential oils of *S. officinalis* and *S. triloba* (table 24).

Table 24: Composition of essential oils of Salviae officinalis folium and Salviae trilobae folium (Blumenthal et al., 2000; Wichtl, 2009).

Essential oils according to the European Pharmacopeia	thujone [%]	1,8-cineole [%]	borneol [%]	bornylacetate [%]
Salviae officinalis folium	25-60	5-15	5-7	max. 2.5
Salviae trilobae folium	5-6	40-60	0.35	No data
Investigated samples				
Salviae officinalis folium IG 008	28.26	15.35	1.85	2.17
Salviae officinalis folium TD 047	22.98	14.04	3.24	3.27
Salviae trilobae folium TD 051	1.5	54.76	3.11	0.97

The monoterpenoids 1,8-cineole and thujone are occurring in significant discriminative levels in both species. The microscopic analysis of Salviae officinalis folium TD 047 and Salviae trilobae folium TD 051 (see 4.1.2.1) additionally confirmed the identity of these samples. Hence, the latter two samples were included in further PCR investigations as reference samples.

4.1.2.3 Multiple sequence alignments of the ITS-regions of Salviae officinalis folium and Salviae trilobae folium

To evaluate, if typic base sequence patterns in the ITS- or ETS-sequence S. officinalis and S. triloba exist, enabling a clear differentiation of both species, a detailed sequence analysis was carried out. To further discriminate the ITS-regions of S. officinalis and S. triloba, multiple alignments were made for all analyzed samples (a section out of the alignments is shown in figure 20). The alignments clearly show high similarities within the group of S. triloba and the group of S. officinalis. The most evident sequence differences are located in the poly G-strand, where S. triloba has insertions of four additional guanines and one additional adenine (figure 20). In the region located in proximity to the poly-G strand there is also an insertion of a guanine and a cytosine that is consistent in all S. triloba samples. Also the slight differences distributed in the whole sequence are coincident for the respective group of species.). In the region located in proximity to the poly-G strand there is also an insertion of a guanine and a cytosine that is consistent in all *S. triloba* samples. Also the slight differences distributed in the whole sequence are coincident for the respective group of species.

The sequencing reaction of the ITS-region of *S. officinalis* and S. *triloba* was very difficult, due to the highly GC-rich sequences (Henke et al., 1997; Sarkar et al., 1990) and especially because of the very long poly G-strand found in both species, even extended in *S. triloba*. During sequencing reaction, the *Taq* polymerase can hardly manage to overcome such poly-G or poly-C regions. Because of the higher bond strength between G and C-bases, the DNA template does not separate successfully during the denaturation step of the PCR. Consequently, in many cases the *Taq* polymerase detaches from the template strand and only a short area of the template strand will be sequenced.

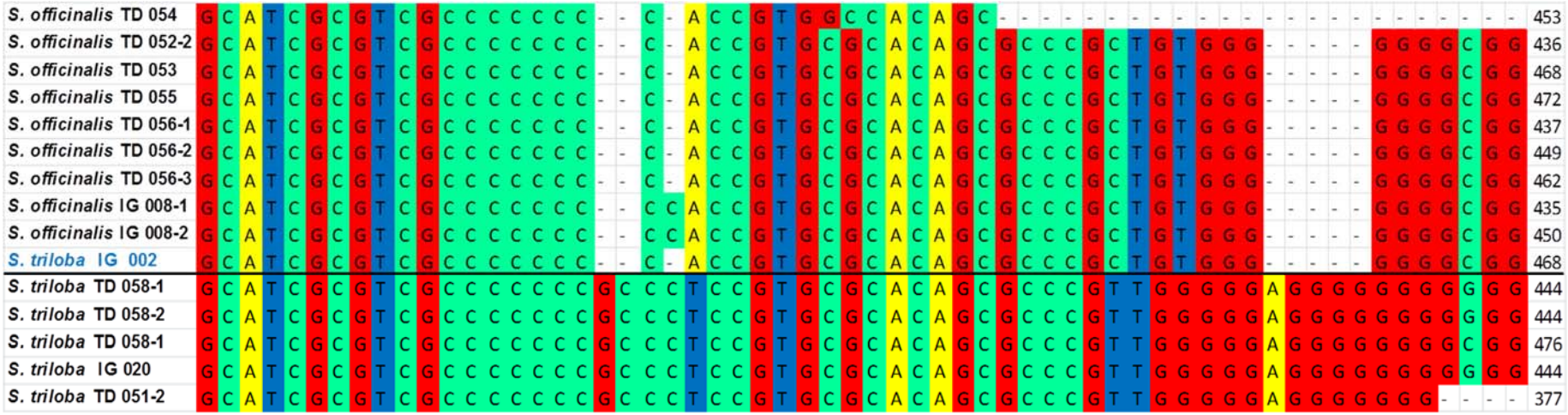


Figure 20: Section of the alignment of analyzed sequences of the ITS region of different samples of *S. officinalis* and *S. triloba* (numbers on the right side of the alignments: respective number of base pairs within the respective sequence alignment).

For this reason, also a part of the ETS-regions of the plant species was sequenced (a section out of the aligned sequences is shown figure 21). For the design of specific primers for the ETS regions of *S. officinalis* and *S. triloba*, available sequence data of ETS regions of different *Salvia* species were selected from GenBank and a sequence alignment of the ETS sequences was made. From the consistent regions, the specific primers were deduced, amplifying a fragment of about 350 bp.

In the part of the ETS-region there is no major difference between the sequences of *S. officinalis* and *S. triloba*, but small differences are distributed along almost the whole sequence strand. In total, there are ten bases that are different when comparing the ETS-regions of both plants. These differences are very constant for both plant species.

The sequencing of the ETS region of the *Salvia* species is easier to perform and results in precise information allowing a clear differentiation of *S. triloba* and *S. officinalis*. This result may also be interesting for pharmacies, as here, in many cases *S. triloba* cannot be purchased, because of the difficulties of certification. *S. triloba* is preferred for some indications because of its reduced content of thujone, which, in high doses, acts as a neurotoxin (Pelkonen et al., 2013).

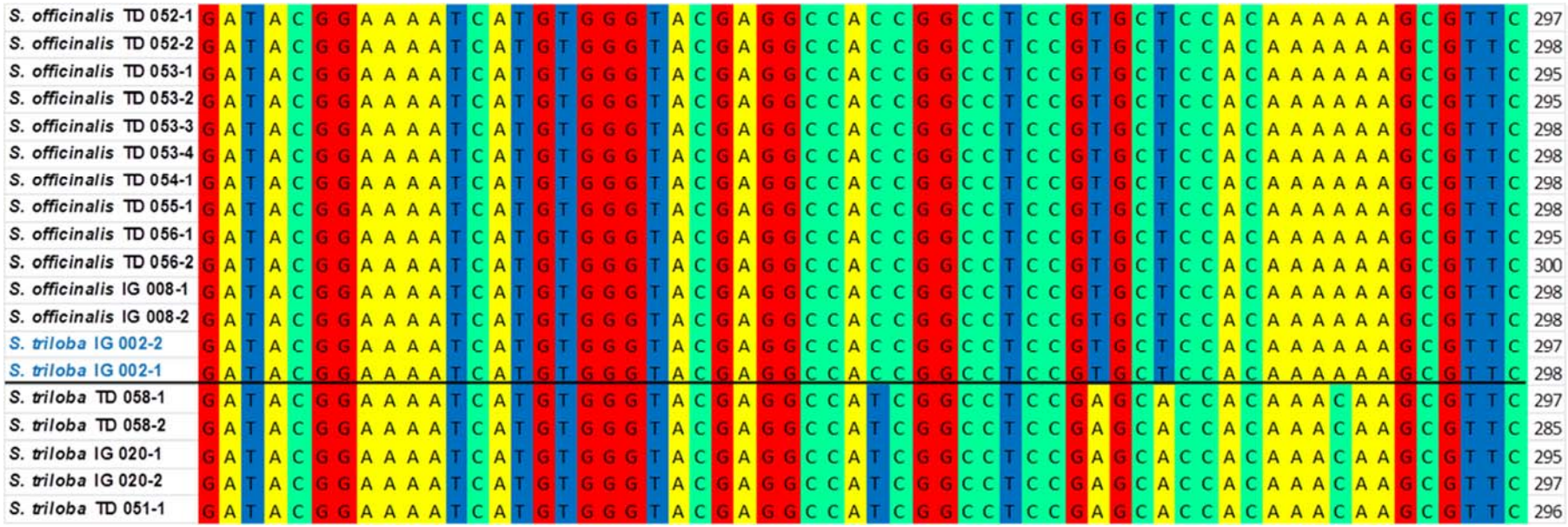


Figure 21: Section of the alignment of analyzed sequences of a part of the ETS region of different samples of *S. officinalis* and *S. triloba* (numbers on the right side of the alignments: respective number of base pairs within the respective sequence alignment).

4.1.3 ITS-barcoding of different species of the Lamiaceae family

Species belonging to the Lamiaceae family selected for the metabolic fingerprinting were also evaluated by DNA barcoding. In total, 31 different plant samples, comprising ten different plant species, were analyzed by this approach.

Bioinformatic evaluation of sequence data from the different Lamiaceae samples using NCBI nucleotide blast alignment showed that almost all herbal species were appropriately identified (table 25). The maximum identity displayed in table 25 corresponds to the extent to which two nucleotide sequences have the same residues at the same positions in an alignment (http://www.ncbi.nlm.nih.gov).

Table 25: List of NCBI nucleotide blast alignment results for DNA sequences of different species of the family Lamiaceae. (Unexpected results shown in blue. Maximum identity [Max. id.]: the extent to which two nucleotide sequences have the same residues at the same positions in an alignment, expressed as a percentage. http://www.ncbi.nlm.nih.gov/books/NBK62051/ 2014)

No.	Species	Origin	Result of NCBI nucleotide blast alignment	Max. id. (%)	Highest homology to accession no. (NCBI database)
1	Marrubbii herba (TD 009), clone 1	pharmacy	*Solanum nigrum subsp. nigrum voucher*	99	FJ980391
2	Marrubbii herba (TD 009), clone 4	pharmacy	*Solanum nigrum subsp. nigrum voucher*	99	FJ980391
3	Marrubbii herba (TD 009), repeat 1	pharmacy	*Marrubium supinum*	96	AF335642
4	Marrubbii herba (TD 009), repeat 2	pharmacy	*Marrubium supinum*	96	AF335642
5	Marrubbii herba (TD 009), repeat 3	pharmacy	*Marrubium supinum*	95	AF335642
6	Marrubbii herba (TD 009) IG 001	gardens of the institute (GPB)	*Marrubium supinum*	96	AF335642
7	Menthae piperitae folium (IG 004)	gardens of the institute (GPB)	*Mentha x piperita voucher* *Mentha spicata*	95 95	JQ230966 DQ667244
8	Menthae piperitae herba (TD 039)	pharmacy	*Mentha spicata* *Mentha x piperita*	*98* *94*	DQ667244 JQ230966
9	Menthae crispae folium (IG 011)	gardens of the institute (GPB)	*Mentha spicata* *Mentha x piperita*	97 93	DQ667244 JQ230966
10	Salviae divinorum folium (IG 007)	gardens of the institute (GPB)	*Salvia divinorum*	99	DQ667249
11	Salviae miltiorrhizae radix et rhizoma EDQM 32482	EDQM	*Salvia miltiorrhiza*	100	EF014345
12	Salviae miltiorrhizae radix et rhizoma EDQM 33040	EDQM	*Salvia miltiorrhiza*	99	KC473245
13	Salviae officinalis folium (TD 047)	pharmacy	*Salvia officinalis*	99	JF301355
14	Salviae officinalis folium TD 052-1	PhytoLab Cultivation [1]	*Salvia officinalis*	99	DQ667225
15	Salviae officinalis folium TD 052-2	PhytoLab Cultivation [1]	*Salvia officinalis*	100	KC473251
16	Salviae officinalis folium TD 053	PhytoLab Cultivation [2]	*Salvia officinalis*	99	KC473251
17	Salviae officinalis folium TD 054-2	PhytoLab Cultivation [4]	*Salvia officinalis*	99	DQ667225
18	Salviae officinalis folium TD 055	PhytoLab Wild harvesting [2]	*Salvia officinalis*	99	DQ667225
19	Salviae officinalis folium TD 056 TKA	PhytoLab Wild harvesting [3]	*Salvia officinalis*	99	KC473251
20	Salviae officinalis folium TD 056 TKB	PhytoLab Wild harvesting [3]	*Salvia officinalis*	99	DQ667225
21	Salvia lavandulifolia, TD 057 TKAfo	PhytoLab Wild harvesting [5]	*Salvia officinalis*	98	DQ667225

No.	Species	Origin	Result of NCBI nucleotide blast alignment	Max. id. (%)	Highest homology to accession no. (NCBI database)
22	Salviae officinalis folium, IG 008 TKB	gardens of the institute (GPB)	*Salvia officinalis*	99	DQ667225
23	Salviae trilobae folium, IG 002	gardens of the institute (GPB)	*Salvia officinalis*	99	KC473251
24	Salviae trilobae folium Galke 1, TD 051	Galke	*Salvia officinalis*	99	KC473251
25	Salviae trilobae folium Galke 1, TD 051, TKBfo	Galke	*Salvia officinalis*	99	KC473251
26	Salviae trilobae folium Galke 2, TD 051, TKAfo	Galke	*Salvia aucheri var. canescens*	99	DQ667286
27	Salviae trilobae folium, NG 1, IG 020 TKBfo	gardens of useful plants (GUP)	*Salvia aucheri var. canescens*	98	DQ667286
28	Salviae trilobae folium, TD 058, TKAfo GATC	PhytoLab Wild harvesting [2]	*1) Salvia aucheri var. canescens* *2) Salvia officinalis*	98 96	DQ667286 DQ667225
29	Salviae trilobae folium TD 058 1 TKBfo Operon	PhytoLab Wild harvesting [2]	*1) Salvia aucheri var. canescens* *2) Salvia officinalis*	98 97	DQ667286 DQ667225
30	Salviae trilobae folium TD 058 2 TKBfo Operon	PhytoLab Wild harvesting [2]	*1) Salvia aucheri var. canescens* *2) Salvia officinalis*	99 97	DQ667286 DQ667225
31	Thymii herba, TD 003	pharmacy	*Thymus vulgaris*	99	EU785939
32	Thymii herba, TD 038	pharmacy	*Thymus vulgaris*	99	EU785939
33	*Thymus vulgaris ssp. citriodorus*, IG 009	gardens of the institute (GPB)	*Thymus vulgaris ssp. citriodorus*	99	EU785939

[1] Eastern Europe country

[2] South-Eastern Europe country 1

[3] South-Eastern Europe country 2

[4] South-Eastern Europe country 3

[5] North African country

One unexpected result was found for *Marrubium vulgare* purchased in a pharmacy (see table 25, no. 1 and 2). According to the result of NCBI blast alignment, the sequence data correspond *to Solanum nigrum* subsp. *nigrum*, a plant containing toxic steroid alkaloids like solamargine, solasonine and solanine (Mohy-ud-Din et al., 2010). As this method is very sensitive, and even very small amounts of plant material are sufficient for DNA isolation, this finding could be explained by an adulteration of the plant sample with the toxic plant. This assumption was affirmed by a repetition of the DNA isolation of this *Marrubium* sample. Three additional, independently taken samples were investigated. According to NCBI BLAST analysis search, the three samples corresponded to *M. supinum*. This species is the closest relative to *M. vulgare*, for which a sequence of the internal transcribed spacer region is available on the database NCBI.

Another contradictory result was found for Menthae piperitae herba (TD 039), purchased in a pharmacy. The sequence data showed highest congruence to a sequence from *Mentha spicata*. The maximum identity for *M. spicata* was 98 %, compared to 94 % for the expected species *M. piperita*.

Concerning the plant species *S. triloba,* the sequencing result for all examined samples was *Salvia aucheri* var. *canescens*, which is due to the fact, that no sequence for *S. triloba* is available at NCBI, and the closest similarity is found to *S. aucheri var. canescens*. Additional analyzes like microscopic and analytical examinations (see 4.1.2.1, 4.1.2.2 and 4.1.3) provided support for the assumption that the samples correspond to the species *S. triloba*. Thus, differences concerning DNA sequences of *S. triloba* are minor but different arguments indicate that the identity of the analyzed *S. triloba*-samples can be confirmed.

4.1.4 Conclusions for the investigations of species belonging to the Lamiaceae with complementary methods

The applicability of metabolic- and ITS barcoding was investigated based on selected plants from the Lamiaceae family. Results from metabolic fingerprinting demonstrated that discrimination of different genera with this method is not a reasonable approach. Due to similar substance classes present in species of different genera, specific classification was not possible. In contrast, a better discrimination of different species from the genus *Salvia* was achieved by this approach.

However, after application of PCA for the discrimination of Salviae officinalis folium and Salviae trilobae folium samples, the identity of the sample Salviae trilobae folium IG 002 remained unclear. Hence, further characterizing methods were applied.

A morphological analysis of the latter sample did not unambiguously reveal the identity of the sample. As another approach, the ITS- and ETS-regions of Salviae officinalis folium and Salviae trilobae folium were investigated, based on a comparison of sequence-alignments. These alignments revealed constant differences between the two species, allowing a clear identification of the sample IG 002 as belonging to the species *S. officinalis*.

4.2 Case study 2: Authentication of herbal substances from TCM

Herbal substances from TCM have a growing acceptance and popularity in western countries e.g. in European countries or the US (Heubl, 2010; Yip et al., 2007; Zhao et al., 2006b). However, there is still a need for appropriate methods for quality control of these drugs (Fan et al., 2012; Joshi et al.; Sahoo et al., 2010; Vlietinck et al., 2009). Therefore, herbal substances from TCM were found an interesting object for applying complementary approaches for quality assessment. In the present work, several herbal substances from TCM provided by the EDQM were analyzed by means of the ITS barcoding approach. Due to statistically not relevant numbers of comparable samples, the metabolomics approach was hardly applicable here (Daniel, 2009). Hence, no data are shown for the metabolic fingerprinting with herbal substances from TCM.

4.2.1 Authentication of herbal substances from TCM by ITS barcoding

A collection of ITS-sequence data from herbal substances derived from TCM has already been started in former PhD studies (Daniel, 2009; Kersten, 2013; Orland et al., 2014). The results from bioinformatic data analysis using NCBI nucleotide blast alignment are displayed in table 26. If DNA isolation was successful, in most cases the labeled species could be confirmed by sequencing and BLAST-analysis results. Also five processed herbal substances were amongst the successfully identified samples, namely Coicis semen, Gardeniae fructus praeparata, Schisandrae chinensis fructus, Zanthoxyli pericarpium and Ziziphi spinosae semen.

The sample of Anemarrhenae rhizoma exhibited the highest similarity with *Coix lacryma*, but the maximum identity of 81% is not a significant value. This may indicate, that we obtained new sequence data, as there are no ITS sequence data for *Anemarrhena aspheloides* and even not for the whole genus *Anemarrhena*. There is one sequence in NCBI that should correspond to ITS data for *A. aspheloides,* but this sequence shows 100% congruence with the fungus *Antherospora vindobonensis* (Accession: JN104591 Name: *Floromyces anemarrhenae*, source host="Anemarrhena aspheloides"). This sequence entry seems to correspond to an adulteration with this fungus, thus, does not correspond to the plant *A. aspheloides.*

Regarding the low number of successfully sequenced samples it is obvious that identification of samples from TCM is difficult (Daniel, 2009; Kersten, 2013). In total,

60 samples were investigated, but only for 31 samples sequence data could be obtained, including five putative adulterations. However, 33 samples were processed, mostly including complex procedures with roasting, boiling, steaming or burning (see table 26, Pao Zhi treatments). Such processing procedures may have led to a degradation of DNA in these samples, thereby impeding successful PCR reactions and sequencing. This assumption is further supported by the fact, that mostly the DNA isolation and subsequent reactions failed for those samples that were processed (see table 26).

From table 26 it can be derived that all investigated *Aconite* species yielded no PCR result, and a DNA measurement also confirmed that DNA isolation was not successful in all cases. *Aconite* species contain toxic diterpene alkaloids, like aconitine, hypaconitine and mesaconitine (Chan, 2009; Niitsu et al., 2013). For reducing these toxic compounds, crude aconite roots have to be decocted for one to two hours (Chan, 2011; Singhuber et al., 2009). This kind of detoxification may lead to a degradation of DNA and be the reason for the failure of the proximate PCR-reaction, as all *Aconite* species investigated here were processed. Similar processing procedures were applied to many other herbal substances investigated here, and in many cases, DNA isolation with standard methodology was not successful (e.g. Anemarrhenae rhizoma, Aucklandiae radix, Gastrodiae rhizoma, Magnoliae officinalis cortex, Rehmanniae radix, Saposhnikoviae radix, Scutellariae radix).

In summary, all adulterations found are native to China (Duke and Ayensu, 1985; Tian et al., 2013; Turner and Fearing, 1964), hence a contamination of the medicinal plants, e.g. during harvesting of the plants seems plausible.

Table 26: List of NCBI nucleotide blast alignment results for DNA sequences of herbal substances belonging to TCM. (Unexpected results shown in blue. Maximum identity [Max. id.]: the extent to which two nucleotide sequences have the same residues at the same positions in an alignment, expressed as a percentage. (http://www.ncbi.nlm.nih.gov/books/NBK62051/ 2014)

No.	EDQM Sample (EDQM code)	Pao Zhi (Common processing procedures of the herbal substances (Körfers and Sun, 2008)	Result of NCBI nucleotide blast alignment	Max. Id (%)	Highest homology to accession no. (NCBI database)
1	Acanthopanacis cortex (32961)	Not common	*Eleutherococcus gracilistylus (syn. Acanthopanax gracilistylus)*	98	FJ980422
2	Acanthopanax giraldii harms cortex (32987)	Not common	*Eleutherococcus gracilistylus (syn. Acanthopanax gracilistylus)*	99	FJ980422
3	Acanthopanacis cortex (32527)	Not common	No result		
4	Aconiti kusneoffii radix preparata (33022)	Zhi Chuan Wu / Sheng Chuang Wu: Boiling until no white areas remain inside the root	No result		
5	Aconiti radix praeparata (32969)	Zhi Chuan Wu / Sheng Chuang Wu: Boiling until no white areas remain inside the root	No result		
6	Aconiti radix praeparata (29242)	Zhi Chuan Wu / Sheng Chuang Wu: Boiling until no white areas remain inside the root	No result		
7	Aconiti radix lateralis praeparata (32994)	Zhi Chuan Wu / Sheng Chuang Wu: Boiling until no white areas remain inside the root	No result		
8	Aconiti radix lateralis praeparata (33032)	Zhi Chuan Wu / Sheng Chuang Wu: Boiling until no white areas remain inside the root	No result		

No.	EDQM Sample (EDQM code)	Pao Zhi (Common processing procedures of the herbal substances (Körfers and Sun, 2008)	Result of NCBI nucleotide blast alignment	Max. Id (%)	Highest homology to accession no. (NCBI database)
9	Anemarrhenae rhizoma (32483) Zhi Mu	Yan Zhi Mu: Roasting in a wok, over mild fire; spraying with salt solution and continue roasting	*Coix lacryma-jobi var. ma-yuen*	81	AB571313
10	Anemarrhenae rhizoma (32964)	Yan Zhi Mu: Roasting in a wok , over mild fire; spraying with salt solution and continue roasting	*Coix lacryma-jobi var. ma-yuen*	81	AB571313
11	Asari radix et rhizoma (32540)	Not common	*Artemisia argyi*	99	GU724270
12	Aucklandiae radix (29254)	Wei Mu Xiang: Heating over mild fire	No result		
13	Aucklandiae Radix (33027)	Wei Mu Xiang: Heating over mild fire	No result		
14	Bupleuri radix (32537) Chai Hu	Soaking with water, cutting and drying	No result		
15	Bupleuri radix (32974)	Soaking with water, cutting and drying	No result		
16	Bupleuri radix (33017)	Soaking with water, cutting and drying	No result		
17	Chaenomelis fructus (33051)	Not common	*Eupatorium fortunei*	100	FJ980338
18	Coicis semen (29251)	Chao Yi Yi Ren: Roasting with or without wheat bran	*Coix lacryma-jobi var. ma-yuen*	99	AB571313
19	Coicis semen (33010)	Chao Yi Yi Ren: Roasting with or without wheat bran	*Coix lacryma-jobi var. ma-yuen*	100	AB571313
20	Daturae flos (33002)		*Datura metel*	99	HQ658595
21	Dioscoreae rhizoma (33052)	Not common	*Dioscorea oppositifolia*	100	EU808018
22	Ecliptae herba (32971)	Not common	*Eclipta prostrata*	99	GU724284
23	Ephedrae herba (32940)	Not processed	No result		

No.	EDQM Sample (EDQM code)	Pao Zhi (Common processing procedures of the herbal substances (Körfers and Sun, 2008)	Result of NCBI nucleotide blast alignment	Max. Id (%)	Highest homology to accession no. (NCBI database)
24	Ephedrae herba praeparata (32939)	Zhi Ma Huang: Roasting with refined honey	No result		
25	Eupatorii herba (32985)	Not common	*Eupatorium fortunei*	100	FJ980338
26	Eupatorii herba (32499)	Not common	*Eupatorium fortunei*	100	FJ980338
27	Evodiae fructus (32508)	Not known	*Tetradium ruticarpum (syn. Evodia ruticarpa)*	99	EU663544
28	Farfare flos (32518)	Not processed	*Tussilago farfara*	99	EU785941.1
29	Farfare flos (33007)	Not processed	*Tussilago farfara*	99	EU785941.1
30	Farfare flos (33043)	Not processed	*Tussilago farfara*	99	EU785941.1
31	Gardeniae fructus (33031)	Not processed	*Gardenia thunbergia*	99	AJ224833
32	Gardeniae fructus praeparata (33024)	Chao Zhi Zi: Roasting in a wok	*Gardenia thunbergia*	99	AJ224833
33	Gastrodiae rhizoma (26597)	Rootstock is steamed until its done and dried and roasted over mild fire	No result		
34	Gastrodiae rhizoma (33029)	Rootstock is steamed until its done and dried and roasted over mild fire	No result		
35	Houttuyniae herba (32984)	Not common	*Amphicarpaea edgeworthii aeky*	99	AF417012
36	Indigo naturalis authentic (31410)	Extraction with water and chalkmilk; plant parts begin to decompose	*Dichrocephala benthamii*	99	FJ980350
37	Isatidis radix (25847)	Not common	*Isatis indigotica*	100	AF384104

No.	EDQM Sample (EDQM code)	Pao Zhi (Common processing procedures of the herbal substances (Körfers and Sun, 2008)	Result of NCBI nucleotide blast alignment	Max. Id (%)	Highest homology to accession no. (NCBI database)
38	Magnoliae officinalis cortex (32502)	The bark is boiled shortly in water; then: Chao Hou Po/Zhi Hou Po: Soaking in ginger decoction, heating and drying in the sun	No result		
39	Magnoliae officinalis cortex (32949)	The bark is boiled shortly in water; then: Chao Hou Po/Zhi Hou Po: Soaking in ginger decoction, heating and drying in the sun	No result		
40	Magnoliae officinalis flos (32944)	Not common	No result		
41	Lycii fructus (32494)	Not common	*Lycium barbarum Yuanguo*	99	JQ320167
42	Lycii fructus (32989)	Not common	*Lycium barbarum Yuanguo*	99	JQ320167
43	Puerariae lobatae radix (29245)	Not processed	*No result*		
44	Puerariae lobatae radix (32504)	Not processed	No result		
45	Rehmanniae radix (32507)	Sheng Di Huang: Burning over fire until the inside is rendered black	No result		
46	Rehmanniae radix (33012)	Sheng Di Huang: Burning over fire until the inside of the root is rendered black	No result		
47	Rehmanniae radix praeparata (33005)	Shu Di Huang: Steaming with rice wine until the inside of the root is rendered black	No result		
48	Salviae miltiorrhizae radix et rhizoma (32482)	Not processed	*Salvia miltiorrhiza*	99	EF014345

No.	EDQM Sample (EDQM code)	Pao Zhi (Common processing procedures of the herbal substances (Körfers and Sun, 2008)	Result of NCBI nucleotide blast alignment	Max. Id (%)	Highest homology to accession no. (NCBI database)
49	Salviae miltiorrhizae radix et rhizoma (33040)	Not processed	*Salvia miltiorrhiza*	99	EF014345
50	Saposhnikoviae radix	Fang Feng Tan: Roasting in a wok until the surface is rendered black	No result		
51	Scutellaria baicalensis georgi radix (30008)	Jiu Huang Qin: Soaking with wine; roasting to dryness	No result		
52	Scutellaria viscidula radix (30009)	Jiu Huang Qin: Soaking with wine; roasting to dryness	No result		
53	Scutellariae radix (33025)	Jiu Huang Qin: Soaking with wine; roasting to dryness	No result		
54	Schisandrae chinensis fructus (32514)	Zhi Wu Wei Zi/ Cu Wu Wei Zi: Steaming with rice vinegar	*Schisandra chinensis*	98	AB558158
55	Spica prunellae herba (32524)	Not common	*Prunella vulgaris*	99	JQ669130
56	Spica prunellae herba (32986)	Not common	*Prunella vulgaris*	99	JQ669130
57	Zanthoxyli pericarpium (32526)	Chao Hua Jiao: Roasting over mild fire in a wok	*Zanthoxylum coreanum* *Zanthoxylum schinifolium*	98 93	JN226789 GU247238
58	Zanthoxyli pericarpium (32952)	Chao Hua Jiao: Roasting over mild fire in a wok	*Zanthoxylum schinifolium*	99	GU247238
59	Ziziphi spinosae semen (32509	Chao Suan Zao Ren: Short roasting over mild fire in a wok	*Ziziphus jujuba*	99	FJ593183
60	Ziziphi spinosae semen praeparata (33011)	stir-baked	*Ziziphus jujuba*	100	FJ593183

4.3 Case study 3: Authentication of Cimicifugae racemosae rhizoma samples with complementary methods

The authenticity of samples of the herbal substance Cimicifugae racemosae rhizoma from different suppliers was investigated by ITS- and metabolic-fingerprinting. Cimicifugae racemosae rhizoma was investigated in this work due to reports on a potential liver toxicity of this drug (Guzman et al., 2009; Mazzanti et al., 2008; Teschke et al., 2009) and the assumption, that the toxicity may be caused by adulterations (He et al., 2006; Johnson and Fahey, 2012; Naser et al., 2011).

4.3.1 DNA barcoding of Cimicifugae racemosae rhizoma samples

Dried roots hand rhizomes of *Actaea racemosa* were used for DNA extraction according to 3.2.2. *Actaea racemosa* is the plant species used for the herbal substance Cimicifugae racemosae rhizoma. In total, 36 samples were investigated and sequence data were obtained for 30 samples. The results from bioinformatic data evaluation NCBI nucleotide blast alignment are shown in table 27.

Table 27: List of NCBI nucleotide blast alignment results for DNA sequences of Cimicifugae racemosae rhizoma samples from different sources. (Unexpected results shown in blue. Maximum identity [Max. id.]: the extent to which two nucleotide sequences have the same residues at the same positions in an alignment, expressed as a percentage. (http://www.ncbi.nlm.nih.gov/books/NBK62051/ 2014)

Code	Sample	Producer	Result of sequencing	Max. Id (%)	Accession No.
Ci 01	Black Cohosh, powder	Nature´s Bounty	No result		
Ci 02	Black Cohosh, extract	Good´N Natural	No result		
Ci 03	Black Cohosh, extract	Herb Tech	No result		
Ci 04	Motherworth Black Cohosh, extract	Eclectic Institute Inc	No result		
Ci 05	Cimicifuga rhizoma conc.	CAELO	*Actaea racemosa*	99	GQ409510
Ci 06	Black Cohosh-rhizoma Cimicifugae Race 20678	EDQM	*Actaea racemosa*	99	GQ409510
Ci 07	Black Cohosh-rhizoma Cimicifugae Race 20679	EDQM	*Actaea racemosa*	99	GQ409510
Ci 08	Cimicifuga racemosa Roots 26953 1	EDQM	*Actaea racemosa*	98	GQ409510
Ci 08	Cimicifuga racemosa Roots 26953 2	EDQM	*Actaea racemosa*	99	GQ409510
Ci 09	Cimicifuga racemosa Roots 26954		No result		
Ci 10	Schlangenwurzel schwarz 17801 clone 1	PhytoLab	*Actaea racemosa*	99	GQ409510
Ci 10	Schlangenwurzel schwarz 17801clone 2	PhytoLab	*Acer rubrum*	99	AY605460
Ci 11	Schlangenwurzel schwarz 17802 1	PhytoLab	*Morus rubra voucher/* *Morus murrayana*	99 99	HQ144181/ FJ605515
Ci 11	Schlangenwurzel schwarz 17802 2	PhytoLab	*Morus rubra voucher/* *Morus murrayana*	99 99	HQ144181/ FJ605515
Ci 11	Schlangenwurzel schwarz 17802 new isolation 1 clone 1	PhytoLab	*Actaea racemosa*	99	GQ409510
Ci 11	Schlangenwurzel schwarz 17802 new isolation 1 clone 2	PhytoLab	*Actaea racemosa*	98	GQ409510
Ci 11	Schlangenwurzel schwarz 17802 new isolation 2 clone 1	PhytoLab	*Actaea racemosa*	99	GQ409510
Ci 11	Schlangenwurzel schwarz 17802 new isolation 2 clone 2	PhytoLab	*Actaea racemosa*	97	GQ409510
Ci 12	Schlangenwurzel schwarz 17803	PhytoLab	*Actaea racemosa*	100	GQ409510

Code	Sample	Producer	Result of sequencing	Max. Id (%)	Accession No.
Ci 13	Schlangenwurzel schwarz 17804 1	PhytoLab	*Actaea racemosa*	99	GQ409510
Ci 13	Schlangenwurzel schwarz 17804 2	PhytoLab	*Collinsonia canadensis*	98	JN578085
Ci 14	Schlangenwurzel schwarz 17805	PhytoLab	*Actaea racemosa*	99	GQ409510
Ci 15	*Cimicifuga racemosa tot. sample A clone 3*	Bionorica	*Actaea racemosa*	99	GQ409510
Ci 16	*Cimicifuga racemosa conc. sample B clone 1*	Bionorica	*Actaea racemosa*	99	GQ409510
Ci 16	*Cimicifuga racemosa conc. sample B clone 2*	Bionorica	*Actaea racemosa*	100	GQ409510
Ci 19	*Cimicifuga racemosa sample E clone 1*	Bionorica	*Actaea racemosa*	97	GQ409510
Ci 20	*Cimicifuga racemosa sample F clone 1*	Bionorica	*Actaea racemosa*	99	GQ409510
Ci 24	*Cimicifuga racemosa clone 1*	Bionorica	*Actaea racemosa*	99	GQ409510
Ci 24	*Cimicifuga racemosa clone 2*	Bionorica	*Actaea racemosa*	98	GQ409510
Ci 24	*Cimicifuga racemosa clone 3*	Bionorica	*Actaea racemosa*	98	GQ409510
Ci 24	*Cimicifuga racemosa clone 4*	Bionorica	*Actaea racemosa*	100	GQ409510
Ci 27	Black Cohosh ROOT POWDER	Starwest Botanicals	No result		
Ci 28	Black Cohosh ROOT POWDER, Organic, sample 4 clone 1	Starwest Botanicals	*Actaea racemosa*	99	GQ409510
Ci 28	Black Cohosh ROOT POWDER, Organic, sample 1 clone 3	Starwest Botanicals	*Actaea racemosa*	99	GQ409510
Ci 28	Black Cohosh ROOT POWDER, Organic, sample 3 clone 3	Starwest Botanicals	*Actaea racemosa*	99	GQ409510
Ci 28	Black Cohosh ROOT POWDER, Organic, *sample 1 clone 1*	Starwest Botanicals	*Sonchus arvensis*	99	HQ161952

In most cases, authenticity of the samples was verified by molecular barcoding (see table 27). But in particular cases, sequences differed. This applied to samples Ci 10 and Ci 11 (see table 27). In sample Ci 10 *Acer rubrum* (Red Maple, Sapindaceae) was identified. For sample Ci 11, two independent DNA isolations were prepared from the

herbal substance and resulting samples were analyzed. Both were identified according to NCBI nucleotide alignment blast as a species from the genus *Morus*. *M. rubra* and *M. murrayana* (Murray State´s Mulberry, Moraceae) are trees common to Eastern North America. A further DNA isolation was performed for Cimicifugae racemosae rhizoma sample Ci 11, and the DNA-fragment amplified by a PCR with ITS-primers was cloned as described in 3.2.5. The sequencing of the plasmid DNA of four analyzed colonies resulted in the adequate/labeled species *A. racemosa*. Results obtained from additional DNA isolation of sample Ci 11 suggests that the finding of *Morus spec.* in the first DNA isolation of Ci 11 was due to traces of *Morus spec.* in this sample.

The Cimicifugae racemosae rhizoma sample Ci 28, directly purchased from a supplier in the US, was also adulterated. Out of four colonies analyzed, one referred to the species *Sonchus arvensis* (Field Sowthistle, Asteraceae). The DNA isolation of the samples Ci 01, Ci 027 and from ethanolic extracts of *A. racemosa* (samples Ci 02 to Ci 04) was not successful, although it was repeated several times and with different methods for DNA isolation. Failure of DNA isolation in case of samples Ci 01 and Ci 27 may result from a preprocessing that may have degraded the DNA (Bernardo et al., 2007; Shaw et al., 2002). The isolation of DNA from ethanolic extracts has proved to be difficult and strongly depends on the technology used for the production of the extract (Novak et al., 2007).

All adulterations found in the scope of the molecular barcoding of *A. racemosa* samples are typically found in the North American vegetation (Brockman, 1986). *A. racemosa* is native to the eastern United States and mainly collected from plants growing in the wild (He et al., 2006). Accordingly, the adulterations found here seem to be evident, as they share a common habitat, but presumably are only contained in a very small, usually tolerable amount. This assumption applied to the sample Ci 11, whose proper identity could only be proven in the second trial (table 27). The investigation of the samples that were adulterated with *Acer rubrum* (Ci 10) and *Sonchus arvensis* (Ci 028) was not repeated, as the investigation of several clones indicated that also *A. racemosa* was found in these samples.

In summary, the ITS barcoding approach proved to be a suitable and effective approach for the authentication of the herbal substance Cimicifugae racemosae rhizoma. In most cases DNA was readily extractable and substantial adulterations could not be confirmed by repeated investigations. The authenticity of 30 samples out

of 36 analyzed samples investigated in total (36 samples of 21 different original Cimicifugae racemosae rhizomae samples) was confirmed. Beneath the six samples that could not be identified, three samples were ethanolic extracts. Nevertheless, problems arise from the high efficiency of detection with this PCR based approach, which also amplifies minor traces of sample material. The high detection limit is an advantage for small amounts of sample material but an obstacle for the up to 2% of herbal adulterations that are permitted in herbal substances (The content of herbal adulterations must not exceed 2%, unless stated otherwise or except in justified and approved cases; European Pharmacopoeia 2013).

4.3.2 Metabolic fingerprinting of Cimicifugae racemosae rhizoma samples

Herbal contaminations, found in Cimicifugae racemosae rhizoma DNA samples by DNA barcoding, were also investigated using ^{1}H-NMR-spectra of the DCM extracts of Cimicifugae racemosae rhizoma samples evaluated with PCA (figure 22).

Ethanolic preparations of *A. racemosa* (see table 27; samples Ci 02 to Ci 04) were not included in these investigations as they cannot be compared with the other samples.

The results of the PCA analysis of the dichloromethane extracts of all Cimicifugae racemosae rhizoma samples are shown in figure 22. The colouring of the samples in figure 22 is indicating the source of supply of Cimicifugae racemosae rhizoma samples.

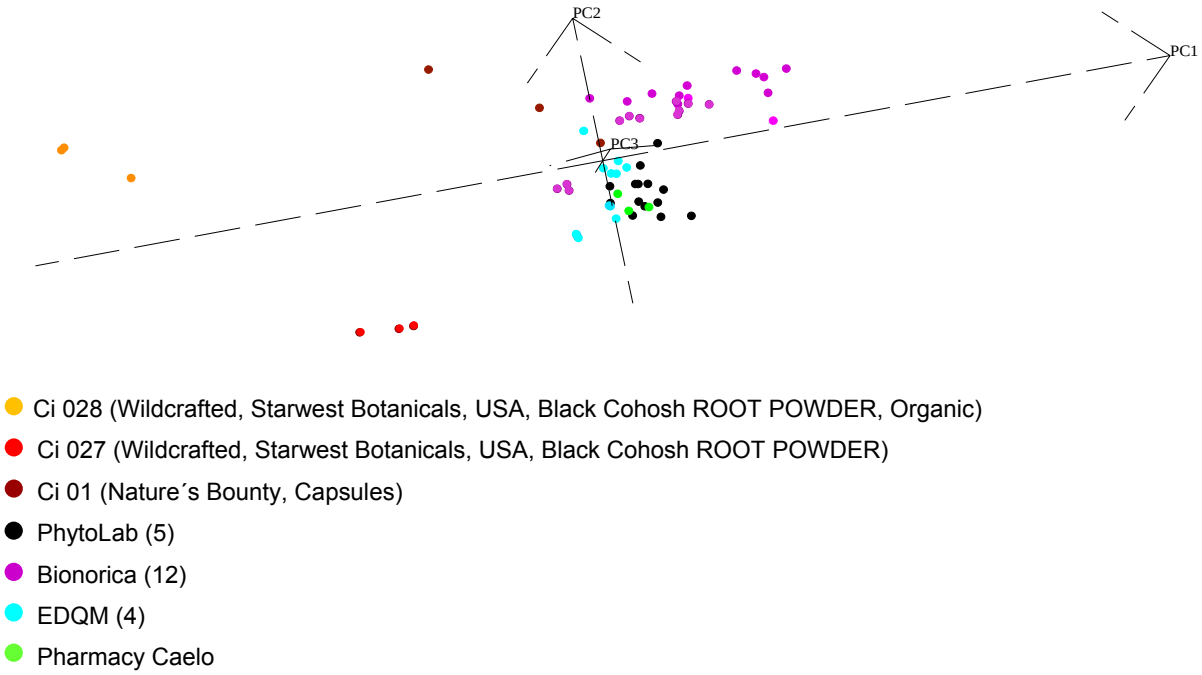


Figure 22: Score plot of PCA for PC1/ PC2 /PC3 of the ^{1}H-NMR-spectra of dichloromethane extracts of different samples of Cimicifugae racemosae rhizoma. Colouring indicating the source of supply. All samples were investigated in triplicates. (In brackets: number of investigated samples)

Samples are discriminated by their source of supply (see figure 22). This applies to samples that were provided by Bionorica, PhytoLab and Caelo and the EDQM. All samples in the main cluster are certified and complying with standards in the European Pharmacopoeia.

Thus, the identity and quality of the outliers Ci 027 and Ci 028 seems to be questionable. They are outliers, forming two independent clusters that are not correlating to each other, although they refer to the same provider (Starwest Botanicals, the only difference in declaration is the addition of “Organic” for Ci 28; see legend of figure 22). Concerning the sample Ci 028, the correct authenticity was already confirmed by the ITS-fingerprint analysis in 4.3.2. Though one adulteration with *Sonchus arvensis* was found for this sample, the DNA of three out of four analyzed colonies corresponded to *A. racemosa* (table 27, sample Ci 028). Here, it has to be verified, if the spectrum of compounds is typical using alternative analytical methods. As the DNA isolation of the samples Ci 01 and Ci 027 was not successful (see 4.3.2), additional analytical methods, for example HPLC investigations are necessary, to further elaborate the authenticity of these samples.

The loadings plot was investigated (figure 23) in order to retrace the classes of compounds, responsible for the discrimination of the samples shown in figure 22.

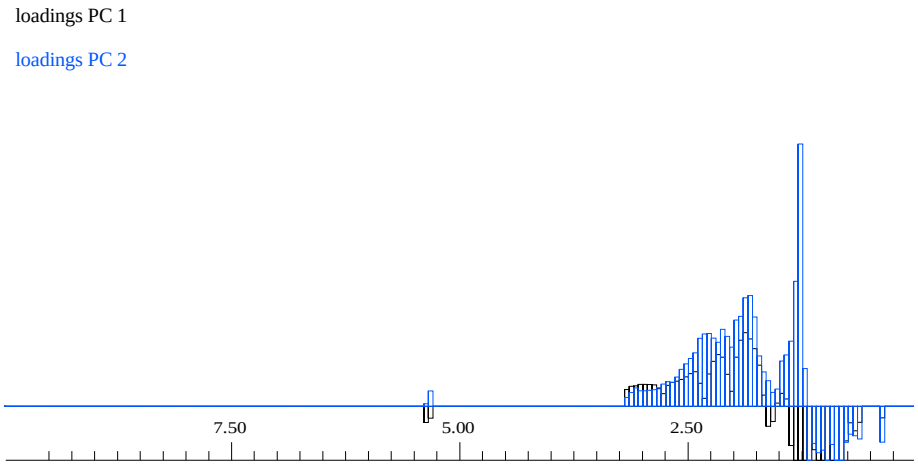


Figure 23: One dimensional loadings for PC1 (blue) and PC2 (black). The scaling corresponds to the chemical shifts [ppm] of the ^{1}H-NMR spectra of *A. racemosa* samples

Secondary metabolites isolated from roots and rhizomes of Cimicifugae racemosae rhizoma are mainly triterpene monoglycosides with a cycloartane skeleton, flavonoids, simple alkaloids and polyphenolic fukiic acid esters (Johnson and Fahey, 2012). The loadings plot in figure 23 demonstrates, that most variance in data is visible in the area between 0 to 2.2 ppm, where protons of terpenoids and steroids resonate. Among more than 40 triterpene glycosides that have been isolated so far from Cimicifugae racemosae rhizoma, the main bioactive triterpene glycosides are actein, 23-epi-26-deoxyactein and cimicifugoside (Chen et al., 2002a, 2002b; He et al., 2006; Johnson and Fahey, 2012; Qiu et al., 2007; Watanabe et al., 2002). Due to their high abundance in Cimicifugae racemosae rhizoma, most variance in the data results from the area where protons of terpenoids resonate, hence the different samples are presumably discriminated by this substance class.

4.4 *Propolis – model system for plant mixtures with unknown content*

Propolis is a challenging substance for metabolic fingerprinting. Its chemical composition may vary considerably depending on its origin. Therefore, unique marker substances for quality control of propolis are not available. The complex multicomponent composition of propolis renders it suitable for a metabolome based approach that is not based on single substances.

Moreover, propolis was chosen as a model system for investigation of herbal mixtures with unknown composition in parallel cooperation with Prof. Chinou from the University of Athens. Her working group performed chemical and microscopic identification of propolis samples. Propolis is a bee product, collected and produced by *A. mellifera*, but it is plant derived and pollen and pieces of vegetative tissues are included during collection of the resinous substance. Hence, there is still plant material in propolis which can be used to analyse the potential of PCR-based methods to retrace the plants serving as source for the production of propolis. In this thesis, an approach with universal primers and another approach with specific primers were investigated (figure 24).

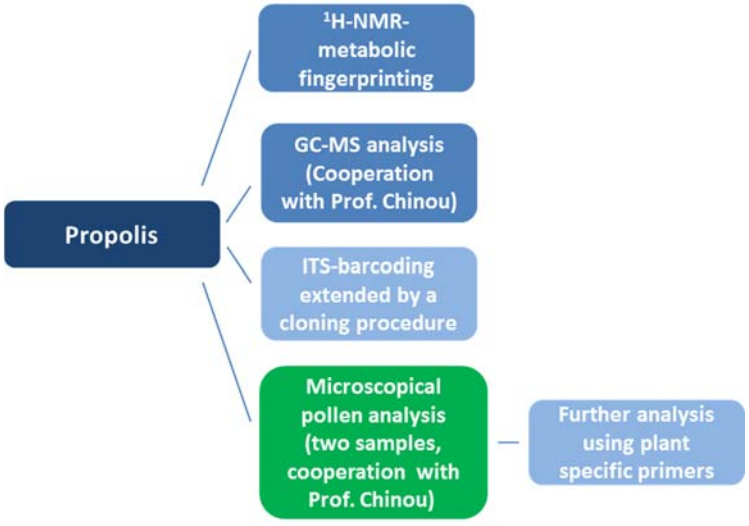


Figure 24: Schematic view of the strategy for microscopical, analytical and PCR-based investigations carried out with propolis samples.

4.4.1 Metabolic fingerprinting of propolis samples

In total, 26 propolis samples from different countries and locations were investigated in a metabolomics approach. A PCA analysis of dichloromethane extracts of the ^{1}H-NMR-spectra of these propolis samples is shown in figure 25.

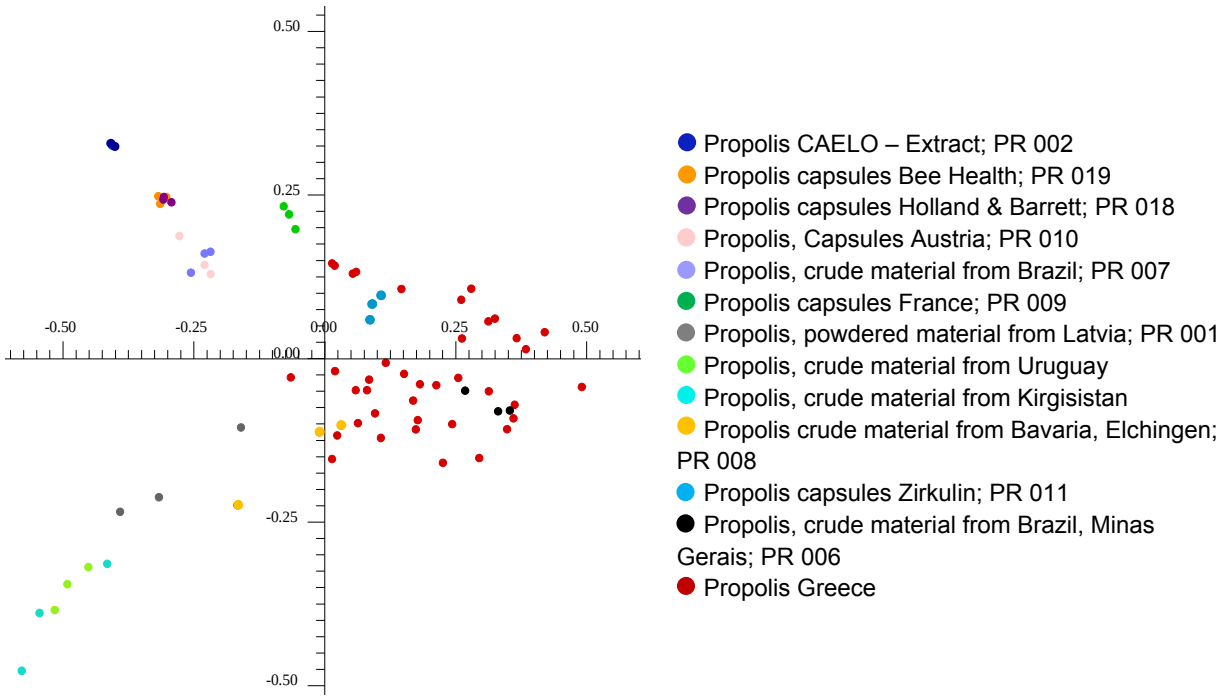


Figure 25: Score plot of PCA for PC1 and PC2 of dichloromethane extracts of the ^{1}H-NMR-spectra of all propolis-samples (Samples investigated in triplicates).

Most of the samples do not necessarily cluster according to their country of origin. Samples from Greece form one diffuse cluster, but some samples from Germany (propolis from Bavaria PR 008, propolis capsules Zirkulin PR 011) and a sample from Brazil, Minas Gerais, can also be found in this cluster. Remarkably, two samples that came from different suppliers but were both purchased from a pharmacy in London, showed great congruence (see purple and orange dots; propolis capsules Bee Health PR 019, propolis capsules Holland & Barrett PR 018). This similarity was already supposed before, because of a similar appearance of the samples and a coincident declaration on the packaging, and seems to be approved by this method.

In general, propolis samples that are preprocessed (the crude extract and the capsules) are more efficiently discriminated by this PCA analysis than crude propolis samples. Crude propolis samples have a more complex composition. The processing of propolis involves a washing process for the removal of extrinsic bees wax. Propolis

is dissolved in ethyl alcohol to eliminate residual wax. In a last step, the propolis tincture is filtered, to remove small foreign particles (preprocessing of propolis: Burdock, 1998).

The loadings plot was evaluated for retracing substance classes that are responsible for the discrimination of the PCA shown in figure 25.

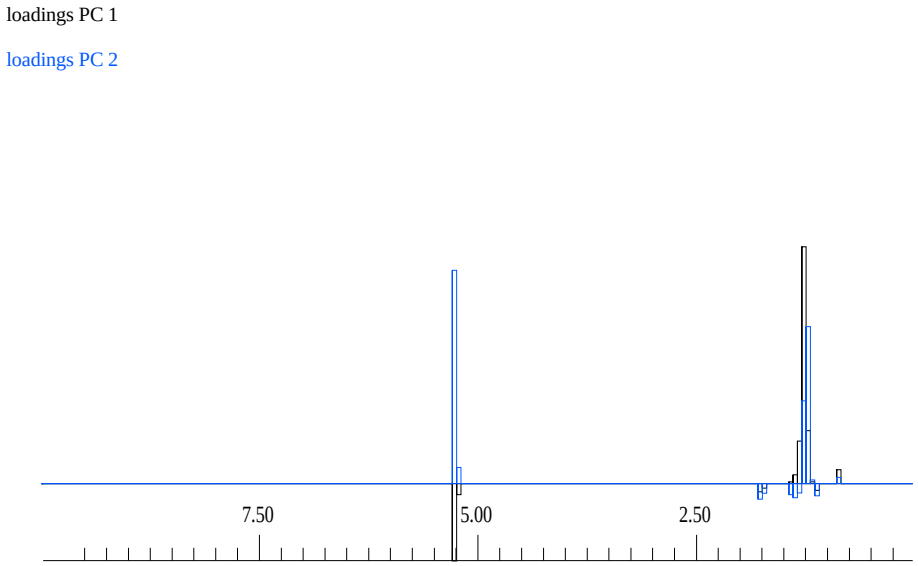


Figure 26: One dimensional loadings for PC1 (blue) and PC2 (black). The scaling corresponds to the chemical shifts [ppm] of the ^{1}H-NMR spectra of dichloromethane extracts of propolis samples.

The loadings plot shows that the main variance of the PCA analysis is obtained from the area around 5.25 ppm and the area around 1.0 to 1.5 ppm. Around the area of 5.25 ppm, protons of flavonoids and phenylpropanoids resonate. Flavonoids like galangin, chrysin, quercetin and pinobanksin are reported to be major pharmacologically active substances of *Populus*-derived propolis (Bankova et al., 2000; Marcucci, 1995), while phenylpropanoids are characterizing, highly abundant compounds of Alecrim-derived propolis (Salatino et al., 2005; Sawaya et al., 2004). The terpenoids β-bisabolol, 1,8-cineole, cymene and limonene, among many others, are reported as components in propolis. Also steroids, such as calinasterol acetate or stigmasterol acetate are present in propolis samples (Marcucci, 1995). Propolis from Greece is reported to be rich in diterpenes (Popova, 2010). Protons of all these components show resonances in the region up to 2.2 ppm.

For six propolis samples, also a GC-MS analysis of the ethanolic extracts of these samples was available through a cooperation with the University of Athens (Prof.

Chinou). Hence, a PCA analysis of these six samples was made, to compare results from the GC-MS analysis with results from PCA analysis.

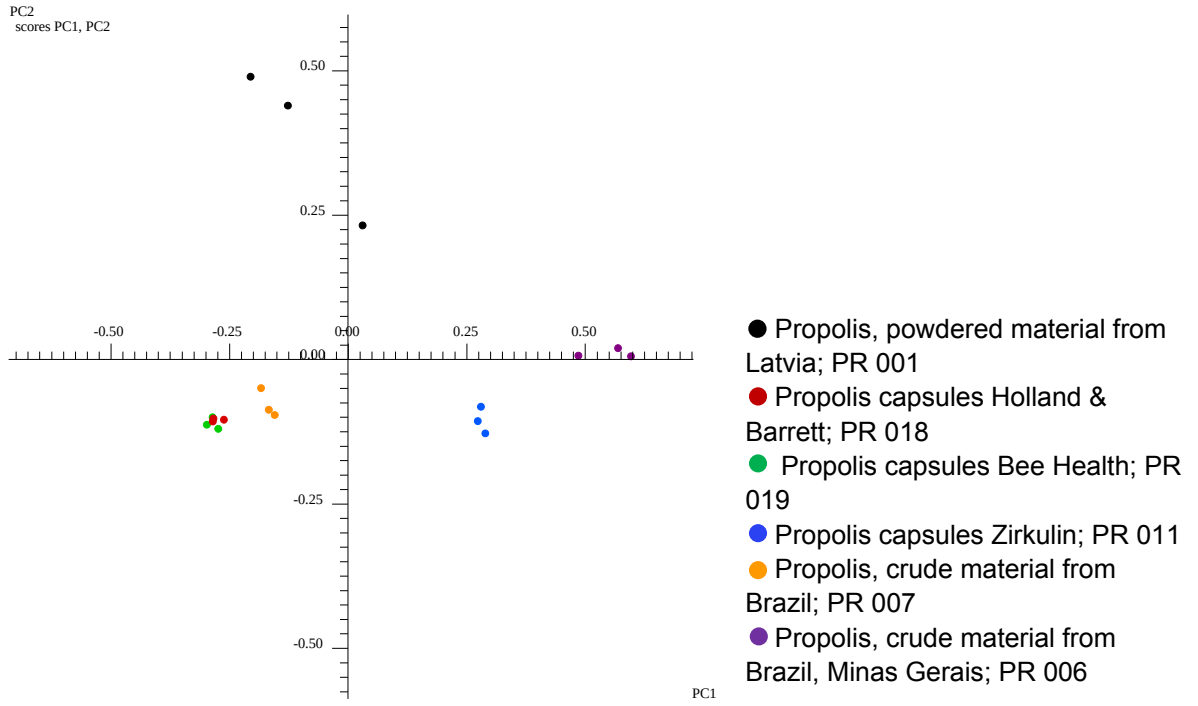


Figure 27: Score plot of PCA for PC1 and PC2 of ^{1}H-NMR-spectra of dichloromethane extracts of six propolis-samples that were also evaluated in a GC-MS analysis. Samples investigated in triplicates.

In the PCA analysis shown in figure 27, the high similarity between the two samples from a pharmacy in England is confirmed (see green and red dots; PR 018 and PR 019). Presumably, the propolis material used for the production of the capsules is the same in the capsules of both suppliers. The two green propolis samples from Brazil (PR 006 and PR 007) do not cluster although they share the same country of origin and, according to the ITS barcoding in 4.4.2, were collected from the same plant source (*Baccaris dracunculifolia*, see figure 29). The chemical composition of green propolis from Brazil is reported to show a broad variation regarding the diversity of classes of secondary metabolites (Park et al., 2002; Salatino et al., 2005).

Again, the loadings plot was investigated, to retrace substance classes that are responsible for classification of samples (figure 28).

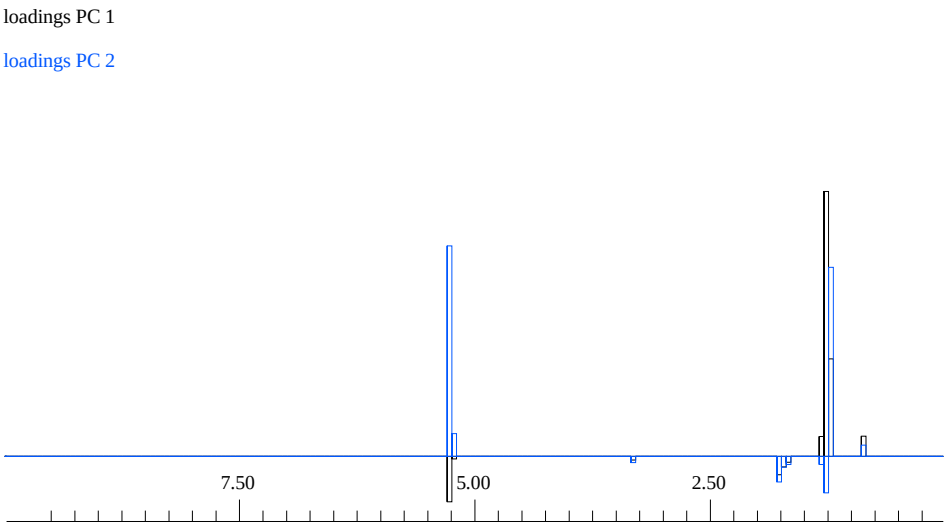


Figure 28: One dimensional loadings for PC1 (blue) and PC2 (black). The scaling corresponds to the chemical shifts [ppm] of the ^{1}H-NMR spectra.

The loadings plot in figure 28 shows high similarity to the loadings plot obtained from the PCA analysis of all propolis samples in figure 26. Again, the main variance of the PCA analysis is obtained from the area of about 5.25 ppm and the area around 1.0 to 1.5 ppm. Since the propolis samples analyzed in this PCA analysis are Poplar-type- and Alecrim-derived propolis samples, which are rich in flavonoids, respectively phenylpropanoids, main variance will presumably be achieved by these substances. They are expected to resonate between 5.0 ppm and 7.5 ppm. Much variance in the data is also obtained from the area between 1 to 1.5 ppm, where the terpenoids and steroids resonate (for a more detailed description see also page 112).

In table 28 the results of a GC-MS analysis of the ethanolic extracts of six propolis samples are presented.

Table 28: GC-MS analysis of ethanolic extracts of six propolis samples (cooperation with Prof. Chinou from the University of Athens).

Chemical compounds [%]	Propolis powder, Germany	Zirkulin propolis capsules, Germany	Bee Health propolis capsules, London	Holland& Barrett propolis capsules, London	Green propolis, capsules	Green propolis, capsules
Aromatic acids	5.66	5.03	22.84	13.54	45.2	45.5
Esters of aromatic acids	13.3	12.41	8.0	20.03	-	-
Flavonoids and chalcones	49.84	25.36	28.84	26.46	-	0.3
Aliphatic acids	1.58	46.38	5.87	5.11	20.4	5.3
Esters of aliphatic acids	1.93	-	11.38	1.07	-	-
Sugars	-	1.59	4.99	6.41	19.5	33.0

When comparing results from the PCA analysis (figure 27, figure 28) with results from GC-analysis, it is very probable that the flavonoids and aromatic acids account for most of the variance of the data. In the poplar-type propolis samples high contents of flavonoids were found by GC analysis. The Alecrim-derived propolis samples contain high contents of aromatic acids. This kind of propolis is reported to be rich in phenylpropanoids. Phenylpropanoids that can also be assigned to the substance class of aromatic acids may be e.g. prenylated cinnamic acid-derived compounds and prenylated coumaric acids, reported for propolis from Brazil. The variance between the two propolis samples from Brazil (PR 006, PR 007) in the PCA analysis (figure 27) may be caused by the differences in the contents of aliphatic acids. The samples Bee Health propolis capsules and Holland & Barrett propolis capsules, both provided by a pharmacy in London had shown great similarities in the PCA analysis of ^{1}H-NMR-spectra of dichloromethane extracts of these samples (figure 25; figure 27). This similarity is not reflected by the GC-analysis of these samples, which displays significant differences in the contents of aromatic acids and esters of aromatic acids. This indicates different resolving power of both methods. Nevertheless, the results obtained by both methods are not contradictory. PCA points out the substances that are responsible for the main variance. From the loadings plot in figure 28 it was concluded that the main variance in the data was obtained from the substance class

of the flavonoids and the contents of flavonoids are also similar for both propolis samples in the GC-analysis.

4.4.2 Analysis of propolis samples by ITS barcoding using universal primers

Furthermore, propolis samples were investigated by a molecular biological approach. Genomic DNA was isolated and an ITS-PCR with universal primers was accomplished. Subsequently the cloning method described in section 1.5.1 was applied.

Approximately 20 colonies for each propolis sample were picked. In some cases, the efficacy of ligation was low, and also with repeated DNA isolations and ligations there were only few colonies analyzable. This was the case for the propolis sample from France (PR 009; 16 colonies picked) and the propolis capsules from the provider Bee Health (PR 019; 12 colonies picked). Then plasmid DNA was isolated and sequenced using universal T7 primer. The sequencing results were investigated using NCBI Blast analysis and sequences with homologies of at least (98 %) are depicted in figure 29. Greek propolis samples were not included in this analysis, as DNA isolation was not successful. All Greek propolis samples were strongly resinous which may have had implications for the DNA analysis und subsequent PCR reactions.

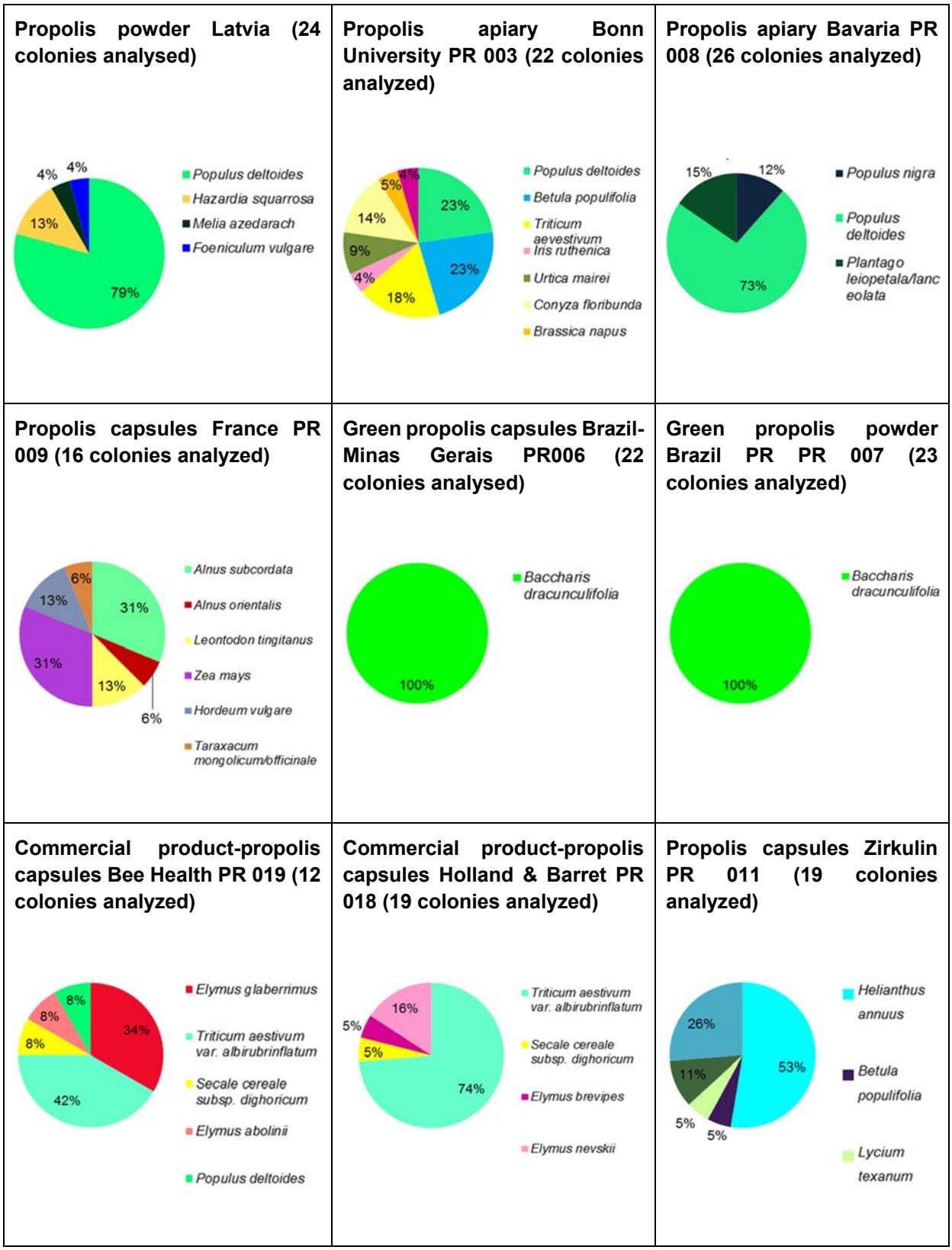


Figure 29: Plant species identified from different propolis samples by DNA barcoding with universal primers und subsequent cloning.

A dominance of *Populus* species can be found in all crude propolis samples originating from Europe. In the case of propolis from Latvia, *Populus* species were found in a ratio

of 79 %, in the propolis sample from an apiary in Bavaria, Elchingen even 85 % and in the propolis sample from the apiary in Bonn the content of *Populus* species was at least 23 %. Besides *Populus* species, other main constituents of European propolis, that are also described in literature, like for example *Betula populifolia* (found in two European Propolis samples) and *Alnus* species (here found in French Propolis) could be identified this method (Burdock, 1998; Marcucci, 1995; Toreti et al., 2013).

The highest diversity of species was found in the propolis sample from the apiary Bonn. Besides *Populus* species, 23 % of all clones investigated were identified as *Betula populifolia* by NCBI blast alignment. Furthermore, the Poaceae *Triticum aevestivum* with 18 % as well as five other species were detected (figure 29, propolis apiary Bonn University). The finding of *Populus* species is in accordance with literature data. *Betula* species were already detected in propolis from Poland (see table 29), that, compared to the flora of Germany, has similar vegetation. In general, literature data about the composition of German propolis are rare.

The only species identified in the two Brazilian propolis samples is *Baccharis dracunculifolia*. This result is supported by literature data, which designate *B. dracunculifolia* as main botanical source of propolis from southeastern Brazil (Teixeira et al., 2005). The origin of one of the two Brazilian samples is South Brazil (Minas Gerais). Another indication for the main botanic source is the greenish-brown color of the samples, which is typical for Alecrim-species derived propolis from this region (Toreti et al., 2013), and both samples investigated had a striking green colour.

Not in accordance with literature data are the findings of species belonging to the plant families Poaceae and Asteraceae (see table 29). The highest percentage of Poaceae and Asteraceae species is found in propolis capsule samples (Propolis capsules France, propolis capsules Bee Health, propolis capsules Holland & Barrett, propolis Capsules Zirkulin). A high proportion of the Poaceae is found in the propolis capsule products bought in a pharmacy in London (see figure 29, propolis capsules Bee Health: 92 % Poaceae; propolis capsules Holland & Barrett: 100 % Poaceae). The more native, non-processed the samples are, less or no Poacees are found in the samples investigated here.

Only *Populus* spp., *Betula* spp., *Alnus* ssp. and *Baccaris dracunculifolia* are mentioned in literature as plant source for propolis. Plants that serve as botanical source for

propolis need to exude resinous material covering leaves or leaf buds. These lipophilic substances are actively secreted by the plants or exuded from wounds (Bankova et al., 2000). For *Populus* species, *B. dracunculifolia* and *Betula verrucosa* literature data on comparative chemical analyzes of propolis and supposed plant source material is available (Bankova et al., 1999, 2000; Salatino et al., 2005; Teixeira et al., 2005), which makes these species reliable plant sources for propolis. The bees collect resin from alecrim (*B. dracunculifolia*) leaf buds and unexpanded leaves (Teixeira et al., 2005). Buds of *Populus*, *Betula* and *Alnus* species supply bees with the resinous substances (Bankova et al., 2000; Marcucci, 1995) needed for producing propolis. For all other plants listed in table 29, no literature data for a possible usage as plant source for propolis is available. Presumably the bees take pollen or other plant parts of these species accidentally. They could “contaminate” their propolis crop unintentionally on their way collecting propolis from their target plants, when they may rest on other plants. Although the bees, that are responsible for the collection of propolis, are specialized for this work (Meyer, 1956), they may get in contact to pollen when they move into their hive or during their collection of propolis. Pollen material collected by honey bees then can stick to the gluey propolis positioned on the legs of the propolis bees (Salatino et al., 2005; personal communications, Dete Papendieck, Apiarist of the Apiary of the University of Bonn).

Table 29: Comparison of plant species, found in propolis samples by ITS barcoding and compared with literature data, refering to plant sources for propolis.

Family	Species found with ITS barcoding	Origin	Literature data
Saliaceae	*Populus deltoides* *Populus nigra*	Bulgaria Albania Europe, Asia, North America	*Populus nigra* (Marcucci, 1995) *Populus nigra* (Toreti et al., 2013) *Populus* ssp. (Bankova et al., 2000)
Betulaceae	*Betula populifolia* *Alnus subcordata* *Alnus orientalis*	Poland Hungary Northern Russia	*Betula, Alnus* spp. (Marcucci, 1995) *Betula* spp. (Marcucci, 1995) *Betula verrucosa* (Bankova et al., 2000)
Asteraceae	*Baccharis dracunculifolia*	Brazil	*B. dracunculifolia* (Bankova et al., 1999; Lemos et al., 2007; Salatino et al., 2005; Toreti et al., 2013)
No literature data available for			
Asteraceae	*Helianthus anuus* *Leontodon tingitanus*		No literature data

Family	Species found with ITS barcoding	Origin	Literature data
	Hazardia squarrosa *Conyza floribunda* *Taraxacum officinale*		
Poaceae	*Elymus brevipes* *Elymus glaberrimus* *Elymus abolinii* *Elymus nevskii* *Zea mays* *Secale cereale subsp. dighoricum* *Triticum aestivum var. albirubrinflatum* *Hordeum vulgare*		No literature data
Apiaceae	*Foeniculum vulgare*		No literature data
Solanaceae	*Lycium texanum*		No literature data
Iridiaceae	*Iris ruthenica*		No literature data
Rosaceae	*Rubus picticaulus* *Rubus caesius x Rubus idaeus*		No literature data
Urticaceae	*Urtica mairei*		No literature data
Brassicaceae	*Brassica napus*		No literature data
Plantaginaceae	*Plantago leiopetala*		No literature data

Especially the high number of pollen from Poaceaes may be an occasional component of propolis, as many of the Poaceaes as well as the Brassicaceae *Brassica napus* are large-scale cultivated and pollen of these plants is widely spread. Additionally, the typical plant and pollen components may be filtered out by the processing procedure (Burdock, 1998). Another reason may be additives that are derived from Poaceae species, mixed with the propolis in the propolis capsules (one additive declared for propolis Zirkulin PR 011 is maize starch; although this plant was not found in the propolis sample Zirkulin PR 011 and the identification of *Zea mays* from maize starch was also not possible with the universal ITS-primers in a previous PhD thesis (Kersten, 2013)).

To prove a potential role of those plants not mentioned in literature in context with the production of propolis, comparisons of the chemical profile of lipophilic secretes of the supposed plants and the propolis samples have to be carried out.

The PCR-approach based on universal primers proved to be a valuable option for retracing plant components in propolis samples. Many species identified are also described in literature as plant sources for the production of propolis. But the PCR-based approach is not suitable for all kinds of propolis samples. No DNA could be isolated out of all Greek propolis samples. A further problem is posed by the amplification patterns of the *Taq* polymerase that may lead to an enrichment of a small number of plant components, but fails to detect other plant components in a sample. Thus, a detection of all plant components in a propolis sample is unlikely. For this reason, also a microscopic analysis of propolis samples in cooperation with Prof. Chinou from the University of Athens (section 4.4.3), as well as another PCR-based approach using specific primers were performed (section 4.4.4).

4.4.3 Microscopic pollen analysis of propolis samples

A microscopic pollen analysis was carried out in cooperation with the University of Athens (Prof. Chinou), to compare the results gained with ITS barcoding of propolis samples with a microscopic analysis of the samples. For this purpose, the propolis sample from the apiary of the University Bonn and the propolis sample from the apiary in Elchingen (Bavaria) were chosen. The results from the pollen analysis of the two propolis samples are shown in table 30.

Table 30: Results from the microscopic pollen analysis (cooperation with Prof. Chinou, University of Athens).

Propolis Bavaria / Elchingen		**Propolis University Bonn**	
Plant Family	**Genus**	**Plant Family**	**Genus/species**
Tiliaceae	*Tilia* sp.	Pinaceae	*Pinus* sp.
Brassicaceae	*Brassica* sp.	Brassicaceae	*Brassica* sp.
Asteraceae	*Ambrosia* sp.	Fagaceae	*Castanea sativa*
	Helianthus sp.	Fagaceae	*Fagus* sp.
	Taraxacum sp.	Tiliaceae	*Tilia* sp.
	Solidago sp.	Gramineae	
	Xanthium sp.	Polygonaceae	*Fagopyrum* sp.
Magnoliaceae	*Magnolia* sp.		
Umbeliferae			
Ericaceae			
Leguminosae	*Trifolium* sp.		
Liliaceae	*Allium* sp.		
Balsaminaceae	*Impatiens* sp.		

The results from the pollen analysis were completely differing from the results achieved by the PCR-approach with universal primers (section 4.4.2). None of the species detected by the PCR approach was confirmed by the pollen analysis. In table 31 results from the pollen analysis of the propolis sample Bavaria / Elchingen (PR008) are depicted in comparison with the results gained by the PCR-approach.

Table 31: Results from the microscopic pollen analysis compared with results from the PCR-approach with universal primers for the propolis sample Bavaria / Elchingen (PR008).

Results from microscopic pollen analysis		Results from PCR with universal primers	
Plant Family	**Genus**	**Plant Family**	**Genus/species**
Tiliaceae	*Tilia* sp.	Salicaceae	*Populus* sp.
Brassicaceae	*Brassica* sp.	Plantaginaceae	*Plantago* sp.
Asteraceae	*Ambrosia* sp.		
	Helianthus sp.		
	Taraxacum sp.		
	Solidago sp.		
	Xanthium sp.		
Magnoliaceae	*Magnolia* sp.		
Umbeliferae			
Ericaceae			
Leguminosae	*Trifolium* sp.		
Liliaceae	*Allium* sp.		
Balsaminaceae	*Impatiens* sp.		

Table 32 demonstrates a comparison of results by microscopic pollen analysis and by PCR using universal primers for the sample propolis University Bonn (PR003).

Table 32: Results from the microscopic pollen analysis compared with results from the PCR-approach with universal primers for the propolis sample University Bonn (PR003).

Results from microscopic pollen analysis		Results from PCR with universal primers	
Plant Family	**Genus**	**Plant Family**	**Genus/species**
Pinaceae	*Pinus* sp.	Salicaceae	*Populus* sp.
Brassicaceae	*Brassica* sp.	Betulaceae	*Betula* sp.
Fagaceae	*Castanea sativa*	Poaceae	*Triticum* sp.
Fagaceae	*Fagus* sp.	Iridaceae	*Iris* sp.
Tiliaceae	*Tilia* sp.	Asteraceae	*Conyza* sp.
Gramineae			
Polygonaceae	*Fagopyrum* sp.		
Pinaceae	*Pinus* sp.		

Interestingly, *Populus* sp., being the most prominent plant source for European propolis, was not confirmed by both pollen analyses, although it was detected in the PCR-approach. In summary, the number of species detected with the pollen analysis was higher than the number of species found by the PCR-approach, maybe due to preferential amplification patterns of the *Taq* polymerase. To investigate, weather specific amplification patterns of the *Taq* polymerase are the reason for which all

species detected with the microscopic pollen analysis were not amplified in the PCR-approach, specific primers were designed to further investigate the propolis samples University Bonn (PR003) and Bavaria / Elchingen (PR008).

4.4.4 Investigation of propolis samples using plant-specific ITS primers

Primers were developed for plant species found in the pollen analysis of the propolis samples for all plants which were at least assigned to the genus level. For pollen samples, where only a plant family could be determined based on the microscopic analysis, no specific primers were designed (Umbelliferae, Ericaceae, Gramineae) as the number of potential plant species is too high. The putative species were identified based on literature describing the flora of Germany (Schmeil et al., 2006), and reasonable plant species were chosen for designing specific primers. For most of the primers a positive control was available (no positive control for *Xanthium strumarum, Ambrosia artemisifolia, Impatiens parviflora*).

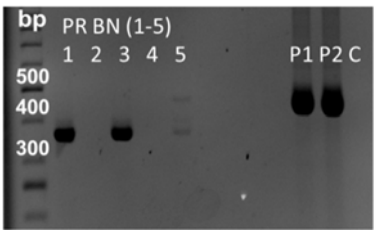
The reference plant samples were either provided by a local pharmacy, the botanical gardens of the Institute for Pharmaceutical Biology in Bonn, or collected in nature and identified by a botanist.

4.4.4.1 Investigation of a propolis sample from the apiary University Bonn (PR 003) with a PCR-based approach using specific primers

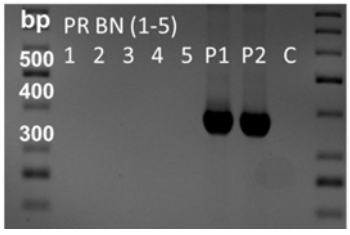
Specific primers were designed for *Pinus sylvestris, Brassica napus, Castanea sativa, Fagus sylvatica, Tilia platyphyllos* and *Fagopyrum esculentum*. The primers were designed based on sequence data of the ITS regions of the different species provided by GenBank. PCR reactions with template DNA of propolis from the apiary of the University of Bonn were positive with amplicons of adequate size for *B. napus, C. sativa, F. sylvatica* and *T. platyphyllos* (see gel documentation figure 30 and table 33). Five independently harvested propolis samples were analyzed from the apiary University Bonn (PR BN 1 to PR BN 5). The propolis samples were selected from different bee hives of the apiary. Table 34 shows the species respectively found in the different propolis samples.

Table 33 Detection of plant species using a PCR-based approach with plant-specific ITS primers from a propolis sample from the apiacy of the University Bonn: Investigated species, the availability of positive controls, and PCR-results.

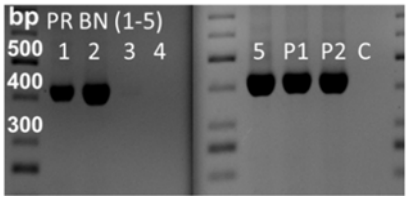
Name	Positive control	PCR-result of the investigated propolis sample
Pinus sylvestris	✓	negative
Brassica napus	✓	✓
Castanea sativa	✓	✓
Fagus sylvatica	✓	✓
Tilia platyphyllos	✓	✓
Fagopyrum esculentum	✓	negative



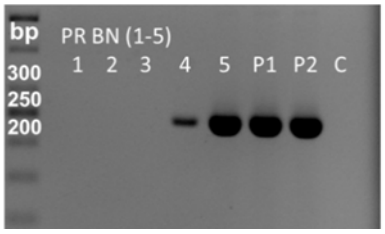
Size of amplicon for *Pinus sylvestris*: 472 bp



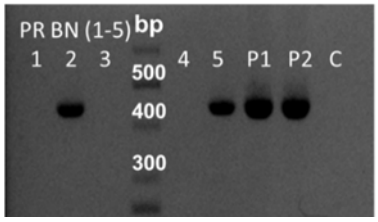
Size of amplicon for *Fagopyrum esculentum:* 374 bp



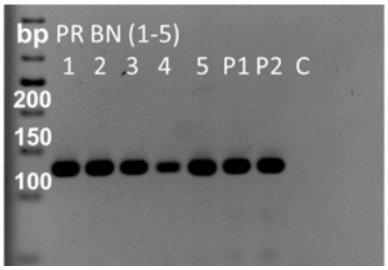
Size of amplicon for *Tilia platyphyllos*: 431 bp



Size of amplicon for *Fagus sylvatica*: 245 bp



Size of amplicon for *Brassica napus*: 455 bp



Size of amplicon for *Castanea sativa*: 123 bp

Figure 30: Propolis apiary Bonn samples investigated with specific primers by a PCR-approach. Lanes 1 to 5: different propolis samples from Bonn (PR BN 1 to PR BN 5); Lanes 6 and 7: the respective positive controls (P1 and P2); Lane 8: negative controls (C).

The detection of *C. sativa*, *F. sylvatica* and *T. platyphyllos* is very interesting, as the bees may also collect resinous material from buds, leaves or wounds from these species. *C. sativa* is also mentioned as possible source for propolis (Bogdanov, 1999), but there is no literature data regarding the usage of *F. sylvatica* and *T. platyphyllos* for the production of propolis. Among the species investigated with specific primers

here, *C. sativa* is the only species found in all five propolis samples from the apiary of the University Bonn (see table 34). This may indicate that *C. sativa* indeed serves as a source for the collection of propolis.

Table 34: Plant species identified with specific primers in the five different propolis University apiary Bonn samples investigated.

Plant species	PR BN 1	PR BN 2	PR BN 3	PR BN 4	PR BN 5
Pinus sylvestris					
Brassica napus		✓			✓
Castanea sativa	✓	✓	✓	✓	✓
Fagus sylvatica				✓	✓
Tilia platyphyllos	✓	✓			
Fagopyrum esculentum					

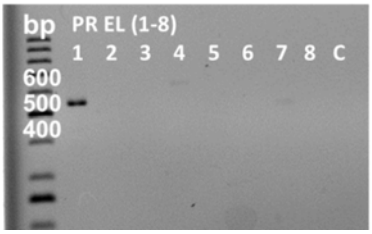
Compared with the results of the ITS barcoding analysis of the propolis sample of the apiary University Bonn (4.4.2), only one common species was detected with both methods (*B. napus*). In case of *P. sylvestris*, it may be, that this species is missing in the barcoding approach with universal primers due to the fact that *P. sylvestris* pine belongs to the group of gymnosperms, thus the universal primers may not be applicable. In general, the species found with the ITS barcoding method are more in consensus with literature data, especially relating to *P. deltoides* and *B. populifolia* (Figure 29). In all crude material propolis samples originating from Europe, a dominance of *Populus* species can be observed (propolis from Latvia: 79 %, Bavaria: 85 % and University Bonn: 23 %). Besides *Populus* species, other leading constituents of European propolis, also described as plant source in literature, e.g. *Betula populifolia* (detected in two European propolis samples) and *Alnus* species (in these investigations detected in French propolis) were identified with ITS-barcoding (Burdock, 1998; Marcucci, 1995; Toreti et al., 2013). However, both methods seem to be complementary and give important hints for the possible plant sources of german propolis.

As there is only rare literature data concerning the composition of German propolis so far, more investigations have to be carried out (see also 4.4.2), to elucidate a potential role of one of the four species found with the specific primers.

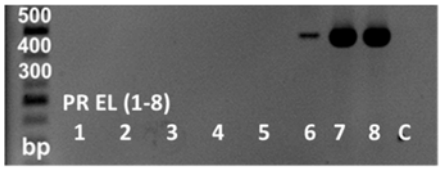
4.4.4.2 Investigation of the propolis sample Bavaria/Elchingen PR 008 with a PCR-based approach using specific primers

As a consequence of the results of the pollen analysis (section 4.4.3), specific primers were designed for the 13 plant species listed in table 35. PCR reactions with template DNA of propolis samples from the apiary Elchingen (Bavaria) were positive with amplicons having the expected size in case of the use of primers for *Brassica napus, Ambrosia artemisifolia, Solidago gigantea and Solidago virgaurea* (see gel documentation pictures: figure 31)

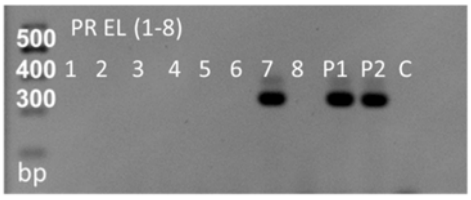
The DNA investigated here was isolated from eight independently investigated propolis samples from the same original propolis sample of the apiary Elchingen.



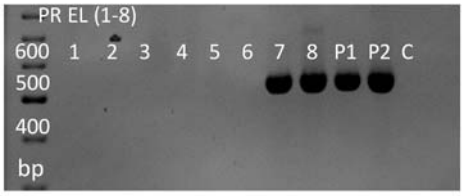
Size of amplicon for *Ambrosia artemisifolia*: 553 bp



Size of amplicon for *Brassica napus*: 489 bp



Size of amplicon for *Solidago gigantea*: 351 bp



Size of amplicon for *Solidago virgaurea*: 507 bp

Figure 31: Propolis Bavaria - Elchingen samples investigated with specific primers in a PCR-approach. Lanes 1 to 8: different propolis samples from Bavaria – Elchingen (PR EL 1 to PR EL8); Lanes 9 and 10: the respective positive controls (P1 and P2; not available for *A. artemisifolia*); Lane 11: negative controls (C) (Only PCRs with positive results for the specific primers with the propolis samples shown).

Table 35: Propolis Bavaria –Elchingen: Species investigated (according to microscopic analysis), availability of positive controls, and respective PCR-results. (n.a.: not available)

Plant species	Positive Control	PCR-Result
Tilia platyphyllos	✓	negative
Brassica napus	✓	✓
Ambrosia artemisifolia	n.a.	✓
Helianthus annuus	✓	negative
Taraxacum officinale	✓	negative
Solidago gigantea	✓	✓
Solidago virgaurea	✓	✓
Xanthium strumarium	n.a.	negative
Magnolia denudata	✓	negative
Trifolium pratense	✓	negative
Trifolium repens	✓	negative
Allium ursinum	✓	negative
Impatiens parviflora	n.a.	negative

Especially *Tilia* and *Magnolia* species seem to be possible candidates for the production of propolis for this sample from the list of species found microscopically, as all verified plant sources for propolis are trees or shrubs. But occurrence of both species could not be confirmed with specific primers in our experiments (see table 35). The finding of plant material from *A. artemisifolia* in the Bavarian propolis sample is interesting, as *A. artemisifolia* is a highly potent allergen (Kanter et al., 2013; Ruëff et al., 2012). It is native to North America, but was introduced to Europe about 100 years ago (Kanter et al., 2013) and is now spreading also in Germany, especially in the south of Germany (Otto et al., 2008). The genus *Solidago* (with the species *S. gigantea* and *S. virgaurea*) so far was not described in literature in context with the production of propolis, hence, the existence of pollen from *Solidago* species in the propolis sample may be judged as an accidental intake of the heavy and gluey pollen. Besides, *Solidago* is a genus of the family Asteraceae, to whom also the species *B. dracunculiafolia* belongs, which is known to be a major botanical source of Brazilian propolis (see table 29).

Table 36: Plant components found with specific primers in the different propolis samples investigated (PR EL 1 to PR EL 8: Propolis Elchingen samples 1 to 8).

Propolis sample	*Ambrosia artemisifolia*	*Brassica napus*	*Solidago gigantea*	*Solidago virgaurea*
PR EL 1	✓			
PR EL 2				
PR EL 3	✓			
PR EL 4				
PR EL 5				
PR EL 6				
PR EL 7			✓	✓
PR EL 8		✓		✓

When comparing the microscopic pollen analysis with the data from ITS barcoding of the propolis sample from Bavaria, no common species was found when applying both methods.

To prove a potential role of those plants not mentioned in literature in context with the production of propolis, comparisons of the chemical profile of lipophilic secretes of the supposed plants and the propolis samples have to be carried out.

4.5 Authentication of herbal substances in defined mixtures and finished herbal medicinal products based on PCR-related methods

Different PCR-based approaches were evaluated as complementary methods for the authentication of plants in mixtures (figure 32). Defined mixtures of herbal substances (DMHS 1 to DMHS 5) were created composed of five defined plant species (table 38). These mixtures of herbal substances and finished herbal medicinal products were investigated using three different approaches as described in the following chapters.

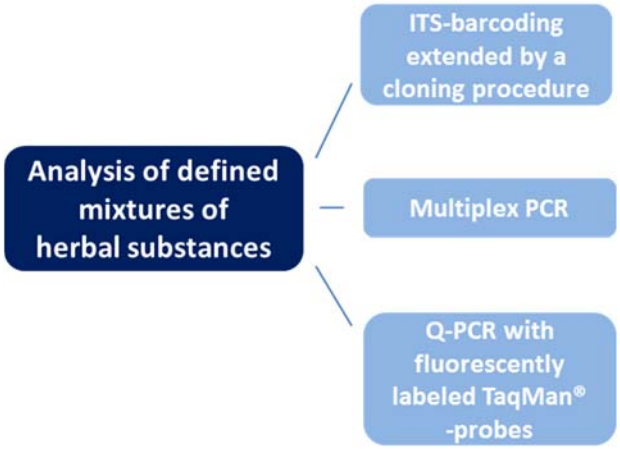


Figure 32: Schematic view of the strategy for different PCR-based approaches for the investigation of defined mixtures of herbal substances and finished medicinal products.

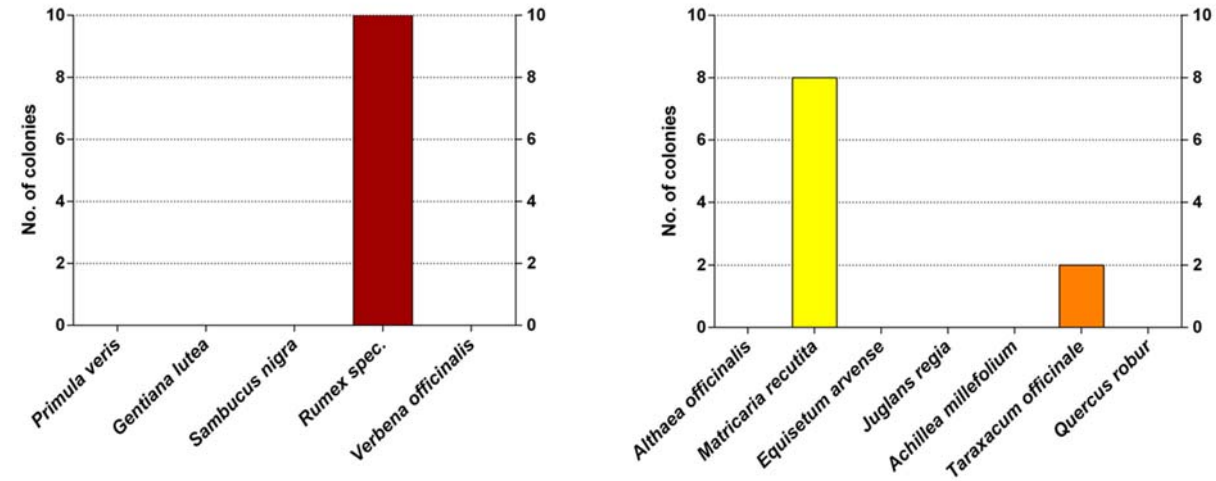
4.5.1 Detection of different herbal components in DMHS by cloning

The cloning method was developed with propolis samples (4.4.2). For evaluating the efficiency of this method, the cloning approach was used for the detection of plant components in DMHS. In preliminary experiments, the herbal medicinal products Sinupret® and Imupret® were investigated. Their composition is shown in table 37.

Table 37: Medicinal plants contained in Imupret® and Sinupret®.

Imupret®	Amount of powdered plant material per pill (mg)	Sinupret®	Amount of powdered plant material per pill (mg)
Althaea officinalis (root)	8	*Primula veris* (flower),	36
Matricaria recutita (flower)	6	*Gentiana lutea* (root)	12
Equisetum arvense (herb)	10	*Sambucus nigra* (flower),	36
Juglans regia (leaf)	12	*Rumex spec*. (herb)	36
Achillea millefolium (herb)	4	*Verbena officinalis* (herb)	36
Taraxacum officinale (herb)	4		
Quercus robur (bark)	4		

For each mixture, ten colonies were selected, plasmid DNA was isolated and sequenced. In case of Imupret®, eight colonies were corresponding to the sequence of *Matricaria recutita*, the sequences of two colonies were identified as *Taraxacum officinale* (figure 23). Referring to Sinupret® all ten analyzed colonies showed analogy with *Rumex sp*. (figure 33). Accordingly, regarding Imupret® only two components out of a mixture of seven plants could be identified and in case of Sinupret® only one plant out of a mixture of five herbal components was detected.



a) b)

Figure 33: Plant components recovered out of Sinupret® (a) and Imupret® (b) by cloning.

This result may be due to the number of colonies that were picked in this preliminary experiment. In addition, the two herbal medicinal products are composed of herbal substance components in varying ratios and numbers. Thus, this method was this time further developed by analyzing at least 50 colonies of a defined mixture of herbal substances.

A defined mixture of herbal substances with equal contents of each plant component was assembled (DMHS 1, see table 38). The mixture was composed of the herbal substances Aristolochiae clematitis folium, Juglandis folium, Matricariae flos, Quercus cortex and Salviae miltiorrhizae radix et rhizoma. Aristolochiae clematitis folium was chosen due to its toxic potential (see 1.2), Salviae miltiorrhizae radix et rhizoma as a representative of TCM-medicinal plants and Juglandis folium, Matricariae flos and Quercus cortex were taken for their general and frequent use as herbal substances and in herbal medicinal products.

The mixtures were set up as shown in table 38 and used for DNA extraction. For mixtures DMHS 1 and DMHS 2, respectively the same portion of each plant was added. For DNA extraction a higher amount of sample material was used in DMHS 2 to investigate the relevance of the amount of starting material for subsequent PCR reactions. Mixtures DMHS 3 to DMHS 5 contained different amounts of each plant (table 38). These mixtures were made to test, if a plant component that is only represented in a plant mixture with i. e. 4 % can be recovered with different PCR-based methods (see also following chapters, 4.5.2 and 4.5.3).

After weighing, plant mixtures were thoroughly ground with the QIAGEN TissueLyser bead mill, and in some cases, an additional milling with mortar and pestle was required. Respectively 20, 40, 60 and 100 mg of plant material were then used for the isolation of DNA. In order to investigate a mixture with equal contents of each plant component only 20 mg samples of DMHS 1 and DMHS 2 were chosen (the other samples were used for the multiplex PCR- (4.5.2) and multiplex-qPCR approach (4.5.3)).

Table 38: Composition of 5 different DMHS: The table shows the amount of plant material respectively used for the assembling of the mixtures (mg / %).

Species	DMHS 1		DMHS 2		DMHS 3		DMHS 4		DMHS 5	
	[mg]	%	[mg]	%	[mg]	%	[mg]	%	[mg]	%
Aristolochiae clematitis folium	36	20	136	20	46	14	302	49	28	4
Matricariae flos	36	20	136	20	31	10	76	12	160	23
Quercus cortex	36	20	136	20	147	45	77	13	47	7
Salviae miltiorrhizae radix et rhizoma	36	20	136	20	50	16	119	19	218	32
Juglandis folium	36	20	136	20	50	15	42	7	230	34

For the defined mixtures the two universal primer pairs TKA and TKB (table 8) were used in separate experiments to amplify the ITS region of the plants. By this means, it was evaluated if the use of different universal primers influences results. Figure 34 exemplarily shows binding sites of the two primer pairs for *Juglans regia*.

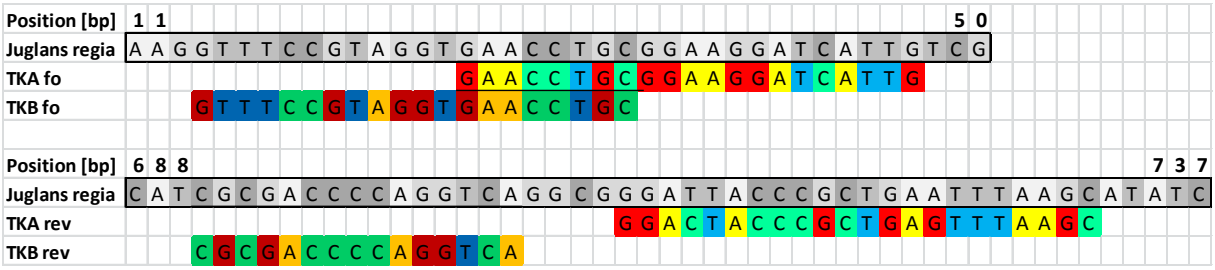


Figure 34: Binding sites for the universal primer pairs TKA and TKB. As template exemplarily the ITS region of *J. regia* (sequence taken from GenBank, accession no. AF399875) was chosen.

Properties of the respective primers like GC contents and template size are shown in table 39. Only plant mixtures DMHS 1 and DMHS 2 were used for this trial, because of the same ratio of plant material for each plant (DMHS 3-5 are analyzed in 4.5.2 and 4.5.3).

Table 39: GC contents and PCR product size for the amplicons generated by the universal primer pairs TKA and TKB for plant species in the defined mixtures of herbal substances (DMHS).

Template	GC % TKA	GC % TKB	Product size primer TKA (bp)	Product size primer TKB (bp)
Juglandis folium	56.3	56.3	706	692
Matricariae flos	51.2	50.9	683	669
Salviae miltiorrhizae radix et rhizoma	62.1	62.0	677	663
Aristolochiae clematitis folium	62.2	64.3	717	669
Quercus cortex	64.0	63.8	652	638

The PCR and cloning steps were accomplished in the same way as described in sections 1.5.1 and 3.2.8. Remarkably, sequencing of plasmid DNA isolated from different colonies gave different results, depending on the use of the primer pairs TKA or TKB.

For each approach (usage of primer TKA or TKB), 25 colonies were analyzed as described in 1.5.1. In case of the use of primer TKA, 22 of the 25 analyzed DNA samples were identified as *M. recutita,* whereas three samples corresponded to *Q. robur*. In contrast, when the primer pair TKB was used, DNA of eight colonies out of 25 colonies analyzed showed the highest similarity to *M. recutita* and 17 colonies were analog to *J. regia* (see figure 35 and table 40).

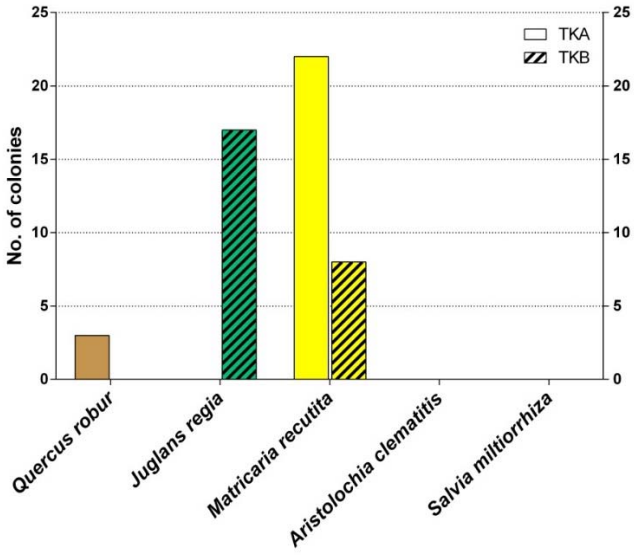


Figure 35: Plant components identified from defined mixtures of herbal substances DMHS 1 and DMHS 2 by cloning. Comparison of the universal primer pairs TKA and TKB applied for PCR and cloning.

Table 40: BLAST search results for ITS sequences obtained for DMHS 1 and DMHS 2 with universal primers TKA or TKB. The total number of analyzed colonies was 25 for each primer pair.

Primer used	No. of colonies analyzed	Highest homology (DNA-level)	Max. identity (%)	GenBank accession number
TKA	22	*M. recutita*	100	EU179212
TKA	2	*Q. robur subsp. robur* *Q. macranthera*	99 99	FM244233 FM244096
TKA	1	*Q. macranthera* *Q. robur subsp. robur*	100 99	FM244096 FM244233
TKB	17	*J. regia*	100	FJ980301
TKB	8	*M. recutita*	99	EU179212

The lack of the detection of *A. clematitis* can be explained by the fact that this plant is a rare example of a species, where the universal primers are not useful for DNA amplification. Thus, no amplification is possible, even not in a singleplex PCR

experiment. This may for example be due to an unfavorable template folding that impedes hybridization of one of the primers or both primers, as the coverage of both primers should theoretically allow amplification of the template. Secondary structures of the template can also be a reason for inefficient elongation by the *Taq* Polymerase (Dutton et al., 1993). *S. miltiorrhiza* could be reliably amplified by the universal primers in former singleplex experiments. Therefore, the absence of this plant could be owing to the possibility, that the other plant DNAs are more abundant in the mixtures, because of a more successful DNA isolation compared to the other plants in the mixture. However, this result may also be due to diverging rDNA copy numbers (Prokopowich et al., 2003; Rogers and Bendich, 1987) in the investigated plants, thus there may be less ITS replicates in the genome of *S. miltiorrhiza.*

As shown in table 39, *J. regia* and *M. recutita*, the plant components that were detected most frequently, indeed have a considerable lower GC content compared to the three other herbal components of the mixture. The GC content seems to have more impact on the success of template amplification than variances in the size of the amplicons, as *Q. robur* has the smallest amplicon sizes, but was only rarely detected (see table 39).

These findings clearly show that a small set of universal ITS-directed primers (here TKA and TKB) are not suitable to detect all components in a mixture of herbal substances. Thus, it is mandatory to increase the number of universal primers or to deduce specific primers providing that the plant components of a plant mixture are known.

4.5.2 Detection of herbal substances in DMHS by multiplex PCR

By means of multiplex PCR, two or more loci are amplified in parallel in a single reaction tube. It was first mentioned by Chamberlain et al. in 1988. In the field of plant science, it was frequently used e.g. for analysis of satellite DNA, typing of transgenic plants, GMO analysis and detection of pathogens (Henegariu et al., 1997).

The multiplex PCR was established for the defined mixtures of herbal substances DMHS 1 to DMHS 5 described in table 38. The multiplex PCR reactions were adjusted and admixed as described in 3.2.8.1. Guidelines for the establishment and optimization

of the multiplex PCR set-up were applied from “Multiplex PCR: Optimization Guidelines” by Innis et al., 1999.

Primer design

An important point when establishing a multiplex PCR-system is a critical design of the competing primer pairs, as the success of the amplification strongly depends on the specificity and selectivity of the primers (Römpler et al., 2006). Additionally, the annealing temperatures of the selected primers have to be homogenous for a simultaneous amplification of all amplicons. All primers were deduced from the ITS-region of the respective plants. First, all target sequences were aligned as multiple alignment to detect the most diverging regions of the different templates for deriving possible candidate primer sequences. Then these regions were screened for functional primers. Another prerequisite of multiplex PCR primers is the gel resolvability of the amplified PCR products (Innis et al., 1999), so the primers were designed such that the products vary in length. A schematic view of the positions of the designed specific primers with expected amplicons is shown in figure 36.

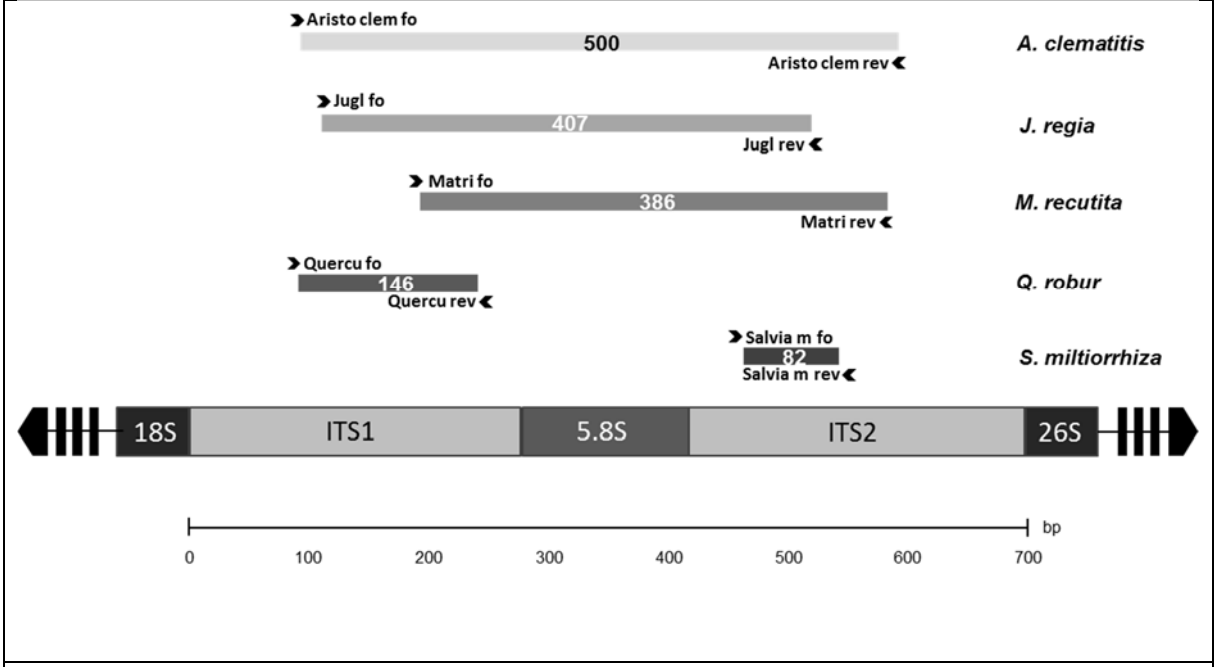
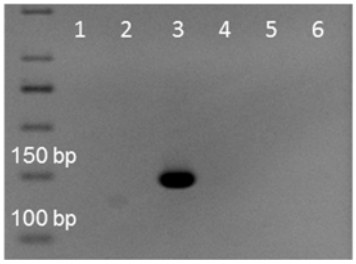


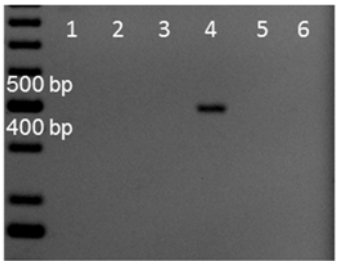
Figure 36: **Schematic view of the nuclear ITS region and the positions of the designed specific primers used for multiplex PCR and resulting amplicons.**

Specificity testing and adjusting of the annealing temperature

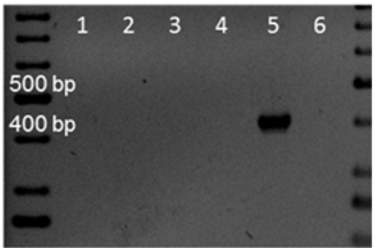
All designed primer pairs were run in a theoretical PCR supplied by the software, with all templates, to initially test the specificity of the primers. If only a single PCR product was obtained when using the adequate template, the primers were subjected to NCBI nucleotide blast analysis. In case all criteria were matching, the primers were purchased and tested first in a singleplex PCR reaction. When the amplification was successful, a gradient PCR for each component and primer was carried out to find one convenient annealing temperature for all primers. Subsequently, the specificity of the primers was tested again by using each DNA template with each primer pair that should be introduced into the multiplex PCR reaction. Only in the case that no amplification occurred with the inadequate templates, the primers were used in the following experiments (see figure 37). This step arose to be the most critical point for the establishment of the PCR.



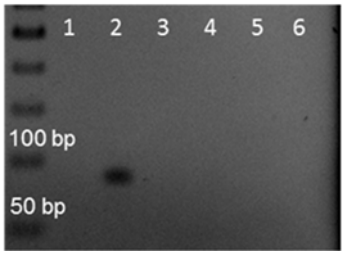
1) Q. robur: 146bp



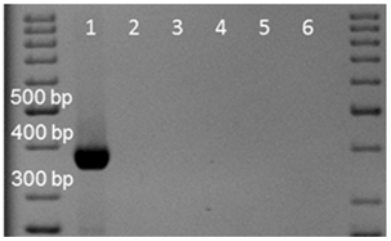
2) A. clematitis: 500 bp



3) J. regia: 407 bp



4) S. miltiorrhiza:82 bp



5) M. recutita: 386 bp

Figure 37: Cross-reactivity check for all plant components of the defined mixture of herbal substances with all specific primers. 1: Master Mix (MM) containing specific primer pair for *Q. robur*, 2: MM with specific primer pair for *A. clematitis*. 3: MM with specific primer pair for *Juglans regia*. 4: MM with specific primer pair for *S. miltiorrhiza*. 5: MM with specific primer pair for *M. recutita*. Lane 1: *M. recutita* DNA, Lane 2: *S. miltiorrhiza* DNA, Lane 3: *Q. robur* DNA, Lane 4: *A. clematitis* DNA,Lane 5: *J. regia* DNA, Lane 6: Control (water).

All multiplex PCR reactions were accomplished as described in section 3.2.8.1. After testing the specificity of the primers, primer concentrations for each target were calibrated for the application in the multiplex PCR run, with primer concentrations

ranging from 50 nM to 400 nM (primer for *Q. robur*: 400 nM, *M. recutita* 50 nM, *J. regia* 100 nM, *S. miltiorrhiza* 200 nM, *A. clematitis* 400 nM). The primer concentrations had to be adjusted according to the efficiency of the primers. For the more abundantly expressed targets, the primer concentrations were limited, until all targets of the mixture were expressed almost equally. (see figure 38).

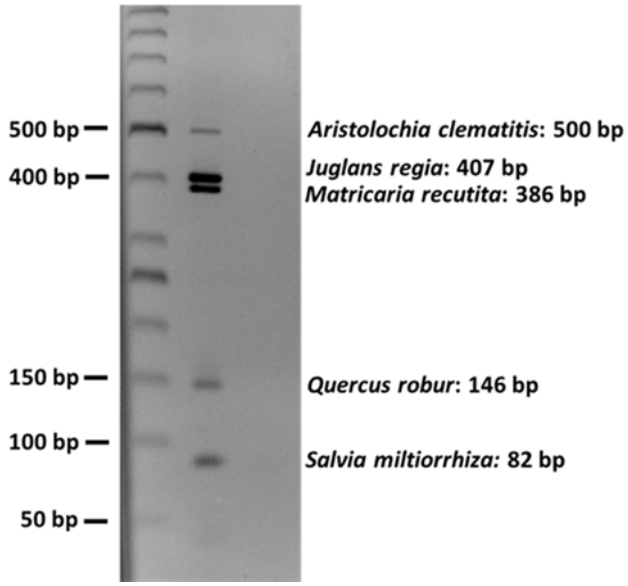


Figure 38: Multiplex PCR with five plant components with adjusted template concentration for each plant species (DNA was separately isolated from each component and then mixed up in a defined concentration).

4.5.2.1 Detection limit of the multiplex PCR approach

For the determination of a detection limit of all components of the mixture, a dilution series of the plant DNAs with sterilized water was made from undiluted to a dilution of 1:10.000 (figure 39) and multiplex PCR reactions were performed with the diluted DNAs (the DNAs were isolated from the single components, then mixed up and diluted). The undiluted DNA corresponded to an amount of 100 ng DNA for each component. As visible in figure 39, the amplicons for *J. regia* (407 bp), *M. recutita* (386 bp) and *S. miltiorrhiza* (82 bp) are visible up to a dilution of 1: 1000, which corresponds to a DNA concentration of respectively 100 pg DNA. *Q. robur* can barely be detected to a final concentration of 1 ng DNA while *A. clematitis* is only detectable with an initial concentration of at least 10 ng DNA.

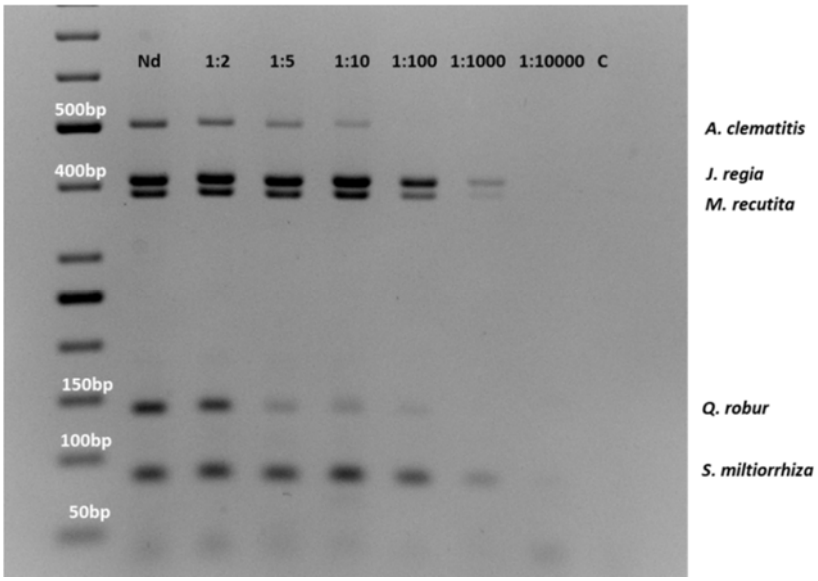


Figure 39: Multiplex PCR with a serial dilution (with water) of DNA isolated out of the single plant components; then mixed for the multiplex PCR reaction. (Fragments: *A. clematitis*: 500 bp, *J. regia*: 407 bp, *M. recutita*: 386 bp; *Q. robur*: 146 bp; *S. miltiorrhiza*: 82 bp) Nd: undiluted = 100 ng DNA used for the PCR reaction; 1:2 = 50 ng DNA, 1:5 = 20 ng, 1:10 = 10 ng DNA; 1:100 = 1 ng DNA, 1:1000 = 100 pg; 1:10.000 = 10 pg DNA; C: Control (water)

4.5.2.2 Analysis of DMHS with multiplex PCR

For the establishment of the multiplex PCR approach, several plant mixtures with different proportions of each component were assembled (table 38).

In all experiments 100 ng DNA resulting from the plant mixture was used for the PCR reactions. The primer concentrations were adjusted as described (3.2.8.1). As shown in figure 40, *J. regia*, *M. recutita* and *S. miltiorrhiza* can be reliably detected in all mixtures. *Q. robur* is detectable in mixtures DMHS 1 to DMHS 4. In DMHS 5, *Q. robur* is only represented with 7 % (see table 38) in the starting material for the DNA isolation and the detection by multiplex PCR is not possible anymore.

The 500 bp fragment of *A. clematitis* could not be detected in mixtures DMHS 1, 2, 3 and 5. These results may be due to a more effective DNA isolation of the better detectable plant components. They are, however, also consistent with the results of the determination of the detection limit. Here, *A. clematitis* and *Q. robur* were the

components that had a significant higher detection limit than the other herbal substances of the mixture, although the DNA concentrations of all plants used for the PCR reactions were equal. It may be that both factors play a role.

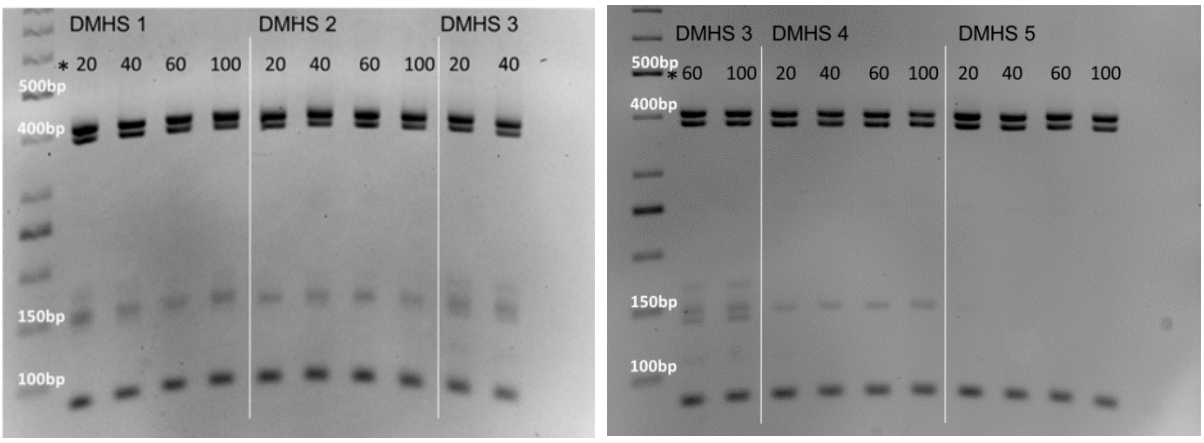


Figure 40: The herbal mixtures DMHS 1 - DMHS 5 (see table 10) investigated with the established multiplex PCR assay. *mg amount of plant material used for DNA isolation. (Fragments: *J. regia*: 407 bp, *M. recutita*: 386 bp; *Q. robur*: 146 bp; *S. miltiorrhiza*: 82 bp)

4.5.3 Detection of different components in DMHS with qPCR in combination with TaqMan® probes

A multiplex qPCR combined with TaqMan® probes was established for the plant mixtures composed of *Q. robur, J. regia, A. clematitis, M. recutita* and *S. miltiorrhiza* (see table 38). Specific primers and probes were purchased from a commercial provider (TIB MOLBIOL GmbH, respectively 2 primer-pairs for each component).

4.5.3.1 Specificity testing of primers and probes used in the qPCR-assay

For specificity testing, each plant component was analyzed alone with each of the five primers and probes in single samples. Only those primers and probes fitting to the targeted DNA sample generated a PCR product (e.g. *J. regia* with primers and probes for *J. regia*). After the establishment of the method, the specificity was tested again, to be able to consider all alterations on methodology. For the final specificity tests, a complete qPCR Master Mix with all primers and probes was used and run under the established conditions. Then all DNAs were added with the exception of the plant component that should be tested for specificity. It was decided to test the specificity in

this way, as each component of the PCR (primers, probes, DNA) influences the PCR process.

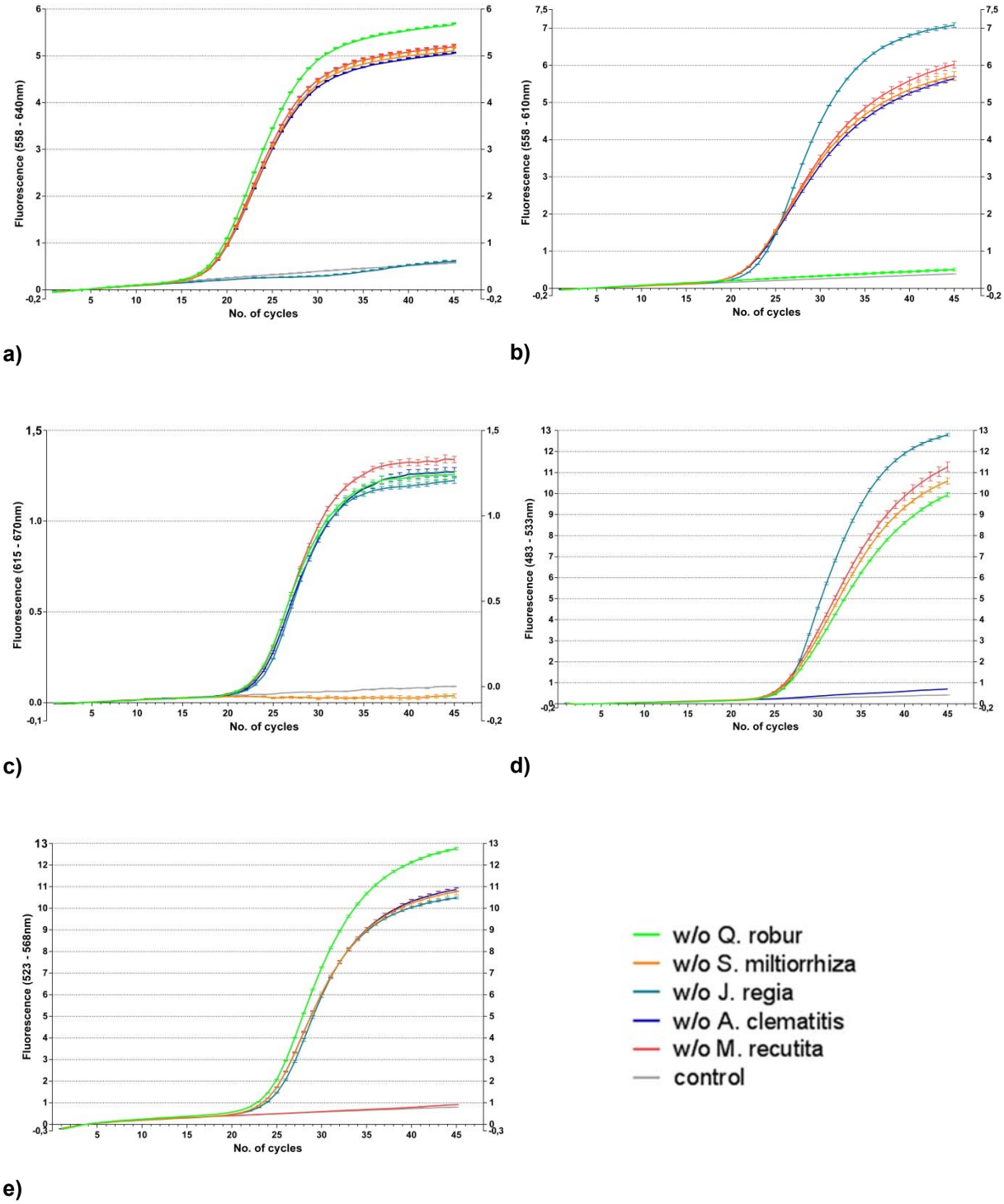


Figure 41: Amplification curves for crossreactivity testing of all primers and probes. Crossreactivity check for qPCR from DNA obtained from a) *J. regia*. b) *Q. robur*, c) *S. miltiorrhiza,* d) *A. clematitis* e) *M. recutita.* Figures a) to e) show the amplification curves obtained at the different fluorescences according to the reporter dye used respectively. All primers and probes and each plant DNA are present in the qPCR-mixture except the DNA of a) *J. regia*. b) *Q. robur,*

c) *S. miltiorrhiza,* d) *A. clematitis* e) *M. recutita*. (Each amplification curve represents the arithmetic mean of triplicate samples)

4.5.3.2 Serial dilution for estimation of the initial DNA concentration

A dilution series was carried out, for estimating the approximate DNA concentration in samples, where the relative amount of DNA of each plant species is not known (see figure 42 and table 41). For this purpose, for each of the five plant components of the defined mixtures (DMHS) 20 ng DNA was added to the PCR reactions. The dilutions of DNA were made by mixing the DNA with sterilized water in a 10-fold range from 1:10 to 1: 1.000.000 (see material / methods). *A. clematitis* and *S. miltiorrhiza* were detectable up to a dilution of 1:1000, corresponding to a DNA concentration initially used for PCR of 20 pg DNA (table 41). *M. recutita, Q. robur* and *J. regia* were traceable up to a dilution of 1:10.000, conforming to a DNA concentration of 2 pg DNA initially used for the PCR reaction. The DNA concentrations were derived from a standard curve equation provided by the Light Cycler Software.

The quantification Cycle (Cq-) values obtained from the qPCR reactions are shown in table 41. The Cq value is an important parameter in terms of qPCR analyzes that gives information on the amount of template in the DNA sample. The Cq-values rise with decreasing DNA concentrations and should be within a range of 20 to 30, whereby a value of 20 indicates, that the nonspecific background fluorescence was surpassed by a proper target fluorescence at an early stage, thus, the initial concentration of the target DNA was significantly higher than in case of a Cq-value of 30.

Table 41: DNA concentrations and Cq values obtained from the serial dilution of the five components of the defined mixture of herbal substances (DMHS). The table depicts the cq-values obtained in the qPCR analysis for each plant component and the amount of DNA inserted for each reaction of the respective dilution (n.d.: undiluted, 1:10 to 1:10.000).

Dilution	*A. clematitis*		*M. recutita*		*Q. robur*		*J. regia*		*S. miltiorrhiza*	
	Cq	DNA (ng)	Cq	DNA (ng)	Cq	DNA (ng)	Cq	DNA (ng)	Cq	DNA (ng)
n.d.	26.71	20.3067	24.43	19.0067	21.01	19.3800	19.72	19.5067	24.79	19.9667
1:10	29.88	2.0060	27.61	2.1267	24.33	2.1067	22.94	2.0533	28.09	1.9733
1:100	33.10	0.1929	31.10	0.1941	27.83	0.2040	26.31	0.1944	31.37	0.1999
1:1000	36.45	0.0195	34.54	0.0184	31.32	0.0198	29.72	0.0200	34.89	0.0207
1:10.000			38.79	0.0011	35.79	0.0020	33.69	0.0020		

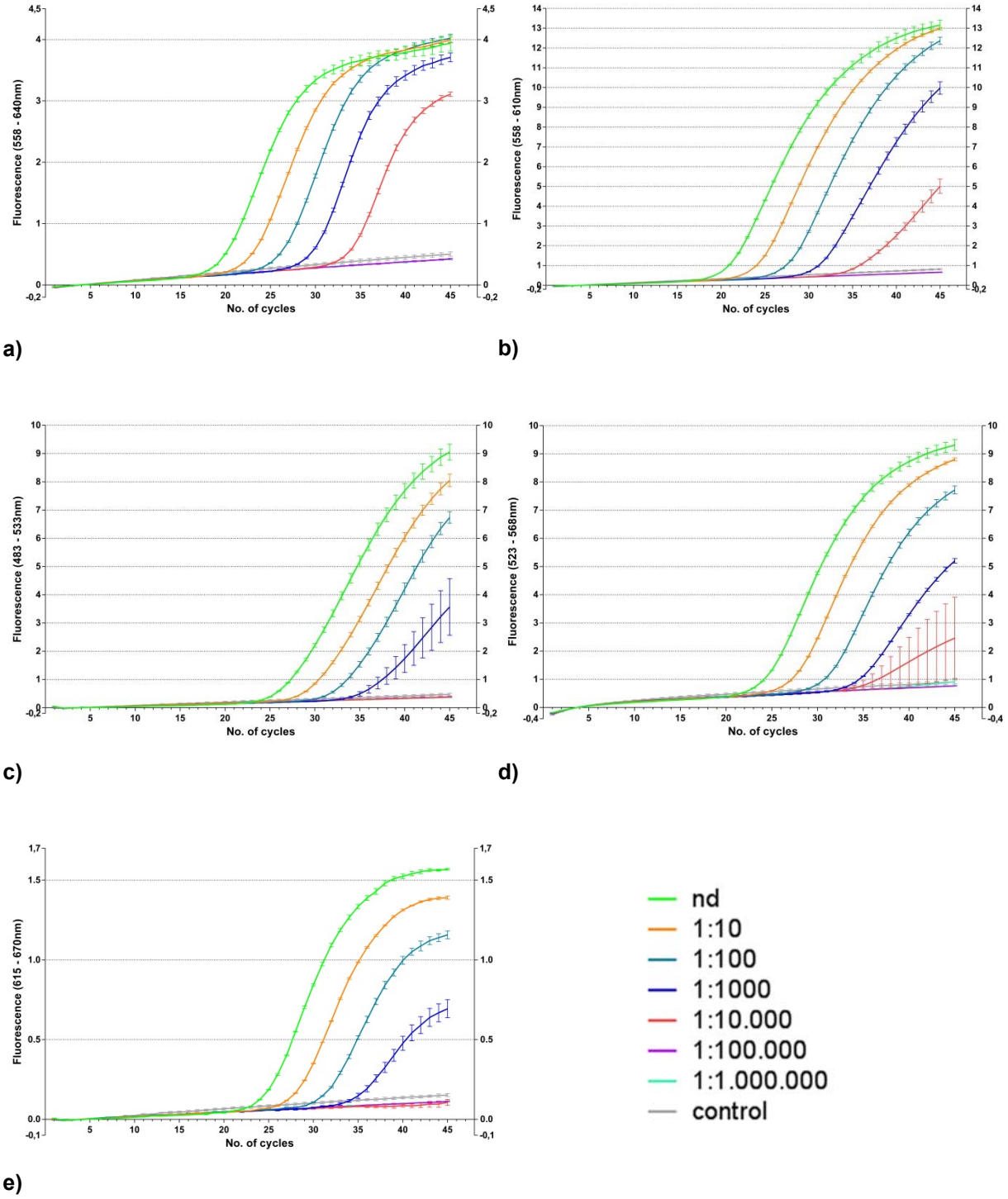


Figure 42: Amplification curves obtained from the qPCR analysis of DNA obtained from a) *J. regia, b) Q. robur, c) A. clematitis, d) M. recutita, e) S. miltiorrhiza* in the serial dilution series for the defined mixture of herbal substances. Figures a) to e) show the amplification curves obtained at the different fluorescences according to the reporter dye used respectively. (Dilution with water, nd: undiluted; Each amplification curve represents the arithmetic mean of triplicate samples)

4.5.4 Multiplex qPCR investigations of DMHS.

Defined mixtures of herbal substances (table 38), were analyzed with the qPCR assay (see figure 43 and figure 44). Figure 44 shows the Cq values obtained when the defined mixtures were investigated. As shown in the Cq-diagram, all five components of the mixture could be reliably verified by qPCR. While in the before mentioned cloning approach (4.5.1) and the multiplex PCR approach (4.5.2) not all components of the DMHS were detected, with the qPCR approach all plants of all mixtures (DMHS 1 to DMHS 5) were traceable.

For the component *A. clematitis,* Cq values in the range of 25.78 and 26.26 were obtained for mixtures DMHS 1 to DMHS 4, while the Cq value for mixture DMHS 5 was considerably higher with 27.86, which correlates to the low amount of only 4% of the plant in this mixture (table 38). *J. regia* in general had very low Cq values, especially in mixture DMHS 5, which corresponds to the high abundance of 230 mg of plant material in this mixture. DMHS 4 shows the highest Cq values for *J. regia* with a Cq of 22.54, conforming to the low percentage of 7% of plant material of *J. regia* in mixture DMHS 4. Compared to the Cq values and DNA concentrations from the dilution series (table 41), the Cq value for *J. regia* in mixture DMHS 4 corresponds to a DNA concentration of about 2 ng DNA, which is approximately 10-fold lower than in the other four investigated mixtures. For *M. recutita* Cq values between 22.57 for mixture DMHS 5 and 24.97 for DMHS 1 were obtained. These values correspond to the amounts of plant material, used for the DNA isolation of the samples. The highest Cq value, meaning the lowest DNA concentration obtained, corresponds nearly to the highest DNA concentration of 20 ng DNA used for the dilution series. As described in table 38, the amount of plant material of *S. miltiorrhiza* used for the different mixtures DMHS 1 to DMHS 5 does not vary very much. This correlates to the Cq values obtained for *S. miltiorrhiza* which are in a range of 24.96 (DMHS 1) and 27.04 (DMHS 4). In case of *Q. robur*, the Cq value for mixture DMHS 5 is not considered, as the performance of the qPCR cannot be evaluated, as shown in the amplification curve for *Q. robur* in DMHS 5 (see figure 43). The Cq values for *Q. robur*, are in a range of 22.33 (DMHS 1) to 25.01 (DMHS 4), whereby the high Cq value of mixture DMHS 4 correlates with the low abundance of *Q. robur* plant material used for this mixture (13 %, see table 38).

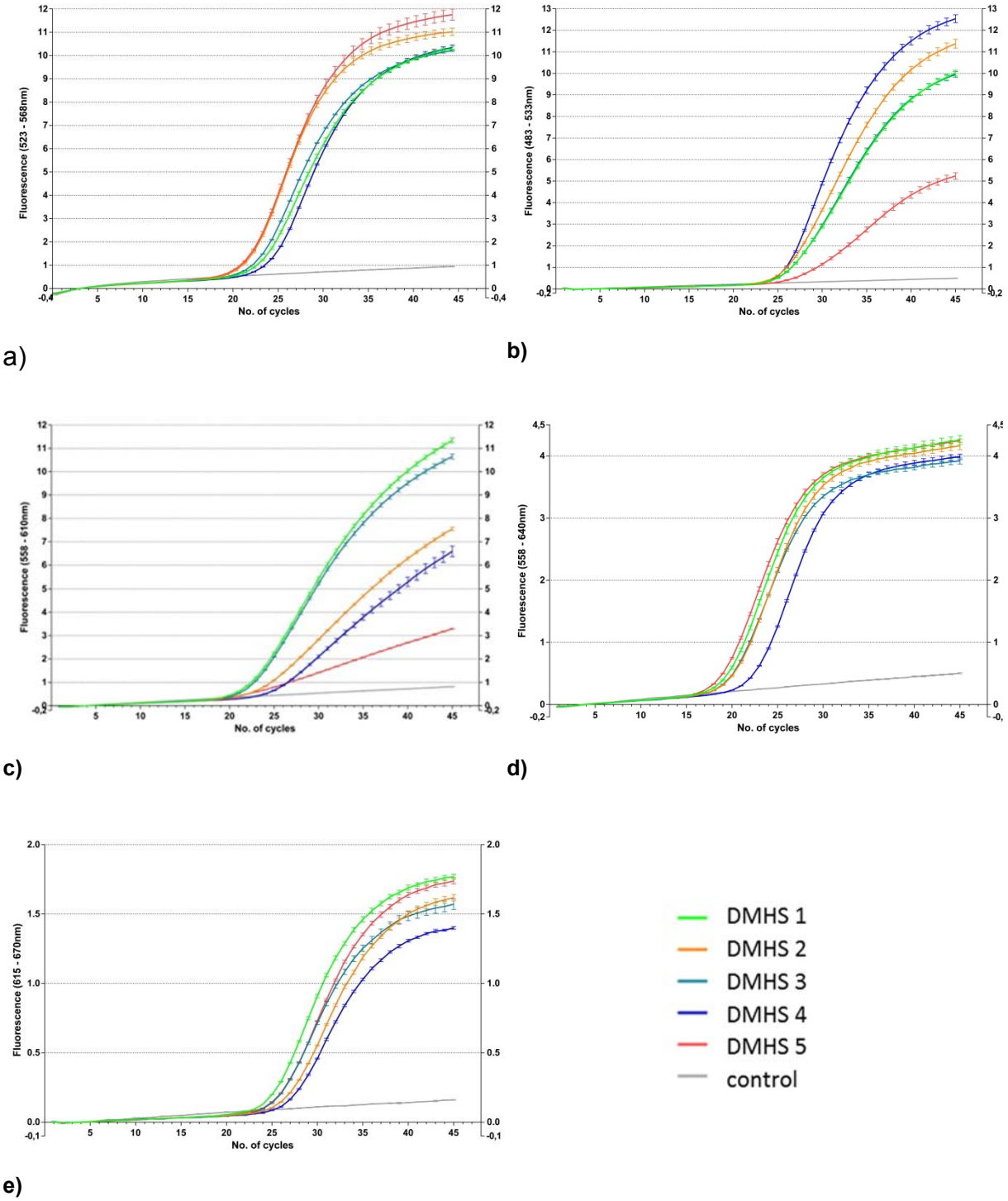


Figure 43: Amplification curves obtained from the analysis of defined mixtures of herbal substances with multiplex qPCR (DMHS 1 to DMHS 5, according to table 10). Figures a) to e) show the amplification curves obtained at the different fluorescences according to the reporter dye used respectively. Detection of a) *M. recutita*, b) *A. clematitis, c) Q. robur, d) J. regia, e) S. miltiorrhiza.* (Each amplification curve represents the arithmetic mean of triplicate samples)

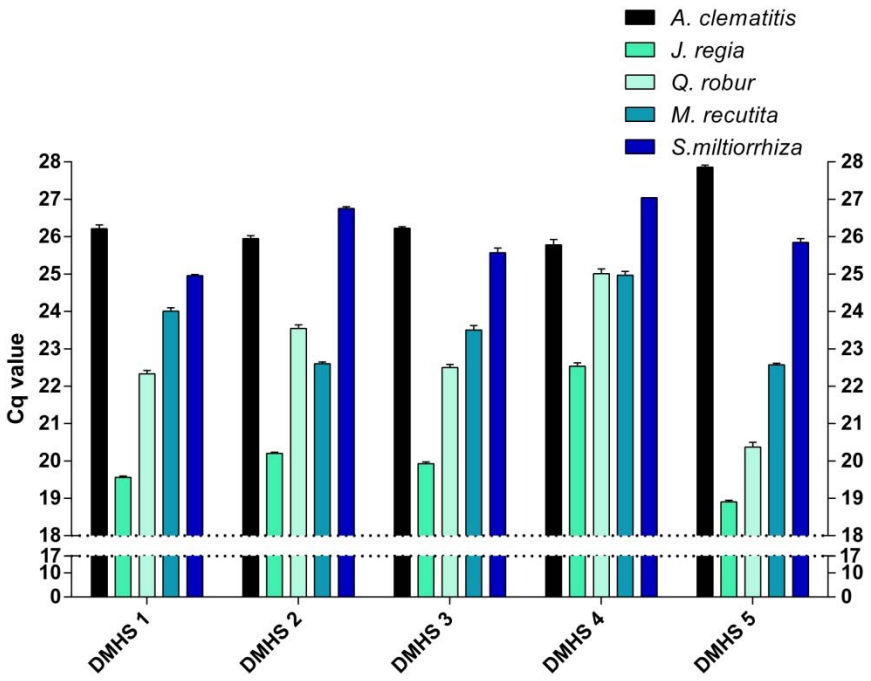


Figure 44: Cq values of the five plant components of defined mixtures of herbal substances (DMHS 1 to DMHS 5, according to table 10) investigated with the established multiplex qPCR assay (Results are depicted on an interrupted y-axis, as the major changes occur with Cq-values starting at a Cq value of about 19; samples investigated in triplicates).

4.5.4.1 Multiplex qPCR investigation of the herbal medicinal product Imupret®

The herbal medicinal product Imupret® was already investigated by the cloning method in part 4.5.1. It is composed of the seven medicinal plants *Matricaria recutita, Althaea officinalis, Equisetum arvense, Achillea millefolium, Juglans regia, Taraxacum officinale* and *Quercus robur.* Consequently, the established multiplex qPCR should only detect the three components *J. regia*, *Q. robur* and *M. recutita.*

As shown in the Cq-diagram in figure 46, the latter three samples were detected in all four Imupret® samples investigated, while *S. miltiorrhiza* and *A. clematitis* were negative. All samples shown here were run with one master mix. The positive control (PC; figure 46) shows signals for the five components of the mixture, while *A. clematitis* and *S. miltiorrhiza* are missing in the analyzed Imupret® samples. *J. regia*, *Q. robur* and *M. recutita* show relatively similar and constant levels of amplification in all Imupret® samples, whereby *Q. robur* exceeds the threshold above the background

fluorescence lately, with a Cq value around 30. This means that only minor concentrations of *Q. robur* DNA are detectable in the investigated samples. Compared to the Cq values and resulting DNA concentrations obtained in the dilution series in 4.5.3.2 and table 41, a Cq value of 30 corresponds to a DNA concentration of about 20 pg DNA of *Q. robur* in the investigated sample. This result may be due to the fact that the isolation of DNA from bark is more difficult than the isolation from other plant tissues like flowers or leaves. *J. regia* was detectable with Cq values in a range between 23 and 24, which corresponds to DNA concentrations of about 2 ng DNA of this plant in the investigated samples (according to the dilution series in 4.5.3.2). *M. recutita* was the major part in the investigated Imupret® samples with an amount of about 20 ng DNA or more.

Despite the low quantities of the component *Q. robur*, all plant components in the Imupret® samples, that were detectable by appropriate primers in this approach, could be verified reliably by the established multiplex qPCR.

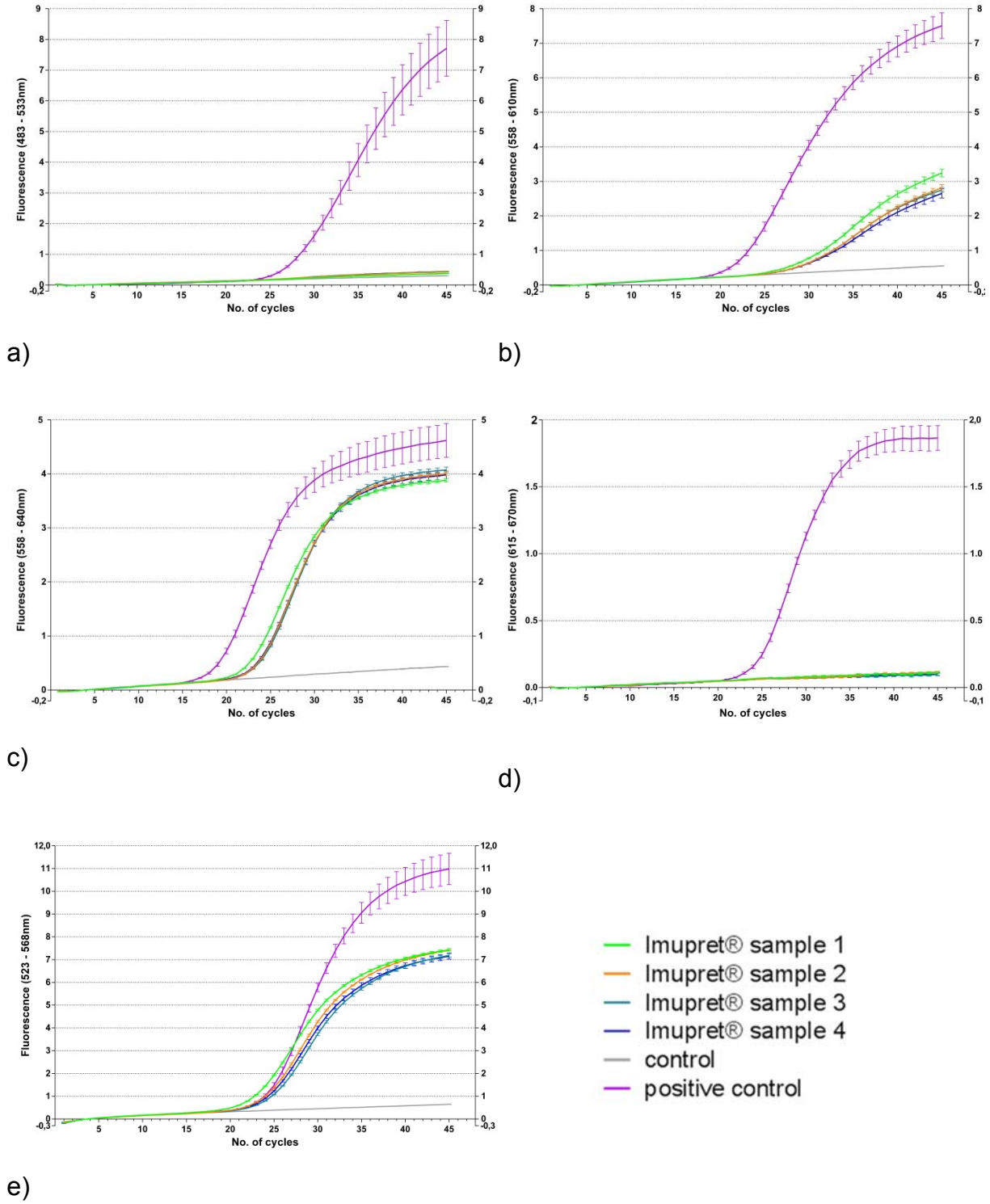


Figure 45: Amplification curves obtained from qPCR investigation of DNA isolated from the finished herbal medicinal product Imupret®. Identification of a) *A. clematitis,* b) *Q. robur*, c) *J. regia,* d) *S. miltiorrhiza,* e) *M. recutita.* Figures a) to e) show the amplification curves obtained at the different fluorescences according to the reporter dye used respectively. Detection of a) *M. recutita*, b) *A. clematitis, c) Q. robur, d) J. regia, e) S. miltiorrhiza.* (Each amplification curve represents the arithmetic mean of triplicate samples)

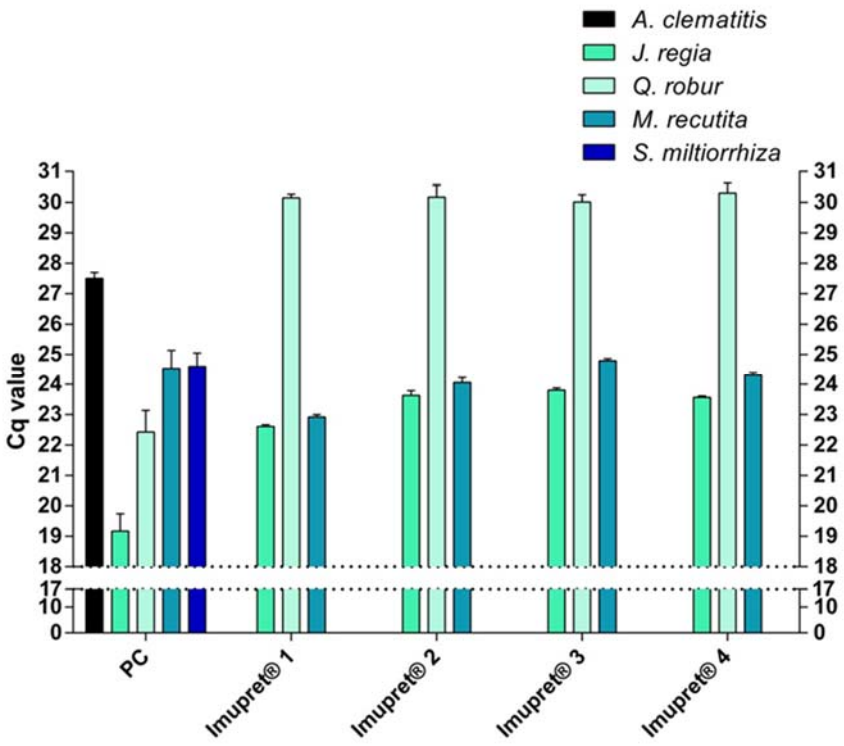


Figure 46: Cq-values of four Imupret® samples investigated with the here established multiplex qPCR assay. PC = Positive Control; Imupret® 1, 2, 3 and 4 represent four independently taken samples from which DNA was isolated with the QIAGEN Plant Mini Kit (Imupret® 1, 2) and the Invitek Stool Kit (Imupret® 3, 4). (Results are depicted on an interrupted y-axis, as the major changes occur with Cq-values starting at a Cq value of about 19; samples investigated in triplicates).

5 Overall Discussion

5.1 Scientific and regulatory framework of the present work

The assessment of quality of medicinal plants is mandatory for ensuring safety and efficacy of herbal medicinal products (Fan et al., 2012; Sahoo et al., 2010; Vlietinck et al., 2009; Zhang et al., 2012). An important step in quality control of medicinal plants is the authentication of the plant species (Zhao et al., 2006b). In this research project various PCR-related methods were investigated that are sought to supplement established pharmacopeial methods. A barcoding approach based on ITS-sequences, already established by Kersten (2013) was used and has been extended by new methods for the investigation of herbal mixtures. Furthermore, a metabolomics approach using ^{1}H-NMR spectroscopy in combination with multivariate data analysis was further evaluated for methodological possibilities and limitations (Daniel, 2009). A special focus of this work was put on the identification of herbal substances in herbal mixtures. Defined mixtures of herbal substances and Imupret® were investigated, to evaluate the potential of the tested methods for finished herbal medicinal products.

5.2 Critical view of the results

5.2.1 Barcoding of herbal substances using universal primers targeting the ITS region

In a previous research project, a method for the authentication of herbal substances with universal primers amplifying the ITS region of plants was established (Kersten, 2013). In the present work, species from the family Lamiaceae, the drug Cimicifugae racemosae rhizoma and plants from TCM were investigated with this approach, to further evaluate the applicability of the method. The ITS region has already been used for the assessment of quality control of medicinal plants (Chiou et al., 2007; Howard et al., 2009; Xue et al., 2007a; Zheng et al., 2009). In this work, it was investigated, if this approach also applies to processed herbal substances or medicinal plants with high contents of essential oils.

In order to investigate species from the Lamiaceae family, 33 different samples, including ten different species, were analyzed. Almost all samples revealed the expected identity. But the high sensitivity of PCR approaches at the same time can create difficulties, as up to 2 % of herbal adulterations that are permitted in herbal substances (The content of herbal adulterations must not exceed 2 %, unless stated otherwise or except in justified and approved cases; European Pharmacopoeia 2013), and minimum amounts of plant material are sufficient for DNA isolation. This was demonstrated by the finding of the adulterant *Solanum nigrum* that was identified in a sample of Marrubii herba (4.1.3). However, all potential adulterants identified by ITS-barcoding could be falsified after repeated analyses of the respective samples that then revealed the expected identities.

A problem with GC rich sequences of *S. officinalis* and *S. triloba* arose. The DNA isolation and subsequent PCR reaction were successful. But the sequencing reaction in many cases was not completed, due to the highly GC-rich sequences (Henke et al., 1997; Sarkar et al., 1990) of *S. officinalis* and S. *triloba*, and especially owing to an extended poly G-strand located in the middle of the target sequence in both species, even extended in *S. triloba*. During sequencing reaction, the *Taq* polymerase can hardly manage to overcome such Poly-G or Poly-C regions. Because of the increased bond strength between G and C-bases, the DNA template does not separate successfully during the denaturation step of the PCR. In many cases, the *Taq* polymerase detaches from the template strand and only a short area of the template strand will be sequenced. Hence, additional primers were deduced from *S. officinalis* and *S. triloba*, enabling a separated amplification of the target sequence, which involved much more efforts. Henke et al. (1997) suggested the admixture of betaine or the addition of a combination of betaine and DMSO to the PCR reaction to ameliorate the amplification of GC-rich sequences. It was supposed that the improvement of PCR-amplification is achieved by decreasing the formation of secondary structures caused by GC-rich regions.

In addition, a part of the ETS-regions of the plant species was sequenced because of the difficulties with ITS-sequencing of the *Salvia* samples. The ETS region was used in plant biology research for phylogenetic purposes several times (Baldwin and Markos, 1998; Bena et al., 1998; Linder et al., 2000; Logacheva et al., 2010; Volkov et al., 1996). The sequencing reaction was successful for *S. officinalis* and *S. triloba*

without the design of additional sequencing primers. The ETS region proved to provide sufficient information for the clear discrimination of *S. triloba* and *S. officinalis,* hence was an appropriate alternative for the highly GC-rich ITS-sequences of the latter two species.

The exceptionally high contents of secondary metabolites that are typic for aromatic plants were supposed to impact DNA isolation by binding nucleic acids (Pirttilä et al., 2001). But this assumption did not apply here.

In the course of the ITS barcoding of species belonging to the Lamiaceae, new sequence data was obtained for *S. triloba*. So far, no sequence data are available for this species in GenBank.

The application of ITS barcoding with regard to Cimicifugae racemosae rhizoma samples also proceeded successfully. The vast majority of samples could be identified by this approach (see table 27). However, a general problem arose with ethanolic extracts of herbal substance. Three ethanolic extracts of Cimicifugae racemosae rhizoma were investigated. Several protocols were applied for DNA isolation but failed to isolate PCR-compatible DNA. The successful isolation of DNA of ethanolic plant extracts is difficult (Kersten, 2013). Novak et al. (2007) developed a procedure, which was intended to enable the isolation of DNA from plant extracts. Minor amounts of intact plant cells found in the extracts were prepared for DNA isolation by a repeated concentration process.

The analysis of herbal substances derived from TCM therapeutic systems by ITS barcoding emerged to be more problematic. Often complex processing procedures are applied here, sometimes resulting in a degradation of the DNA of the herbal substances. In the field of Chinese Medicines the processing of herbal substances (Pao zhi) is a very common step to increase the effectivity, reduce or even eliminate the toxicity or simply alleviate the storage of drugs (Körfers and Sun, 2008; Li et al., 2013). These alterations are achieved by special processing procedures (Table 26, "Pao Zhi"-treatments). Many of these treatments, like e.g. roasting and cooking, have a high potential to promote a degradation of the DNA of the plant material. The isolation of DNA from processed plant samples appropriate for PCR is a known problem especially in the field of TCM (Shaw et al., 2002; Singhuber et al., 2009) or food technology (Bernardo et al., 2007). Nevertheless, many samples were successfully

analyzed and the adequate identity was confirmed, including also samples that were processed (see table 26). To improve the amplification of DNA from strongly processed samples, the usage of primers amplifying a shorter segment (ITS1 or ITS2 alone) was a successful approach in former PhD projects (Daniel, 2009; Kersten, 2013) and was already proposed in literature (Blattner, 1999). Furthermore, repair reaction methods were already developed for highly degraded DNA of herbal substances that are sought to enable a successful amplification of the ITS region for identification purposes (LeRoy et al., 2002)

Overall, the identity of 83 out of 129 samples was confirmed by ITS barcoding. Nearly all samples that could not be identified by this approach were strongly processed herbal substances from TCM. Thus, if applicable, this is a robust, fast, precise and efficient approach, particularly in view of the fact that beneath these samples many herbal substances were dried woody plant parts, in many cases processed. It provides high sensitivity, thereby allowing discrimination even of closely related species, as could be demonstrated here for *S. triloba* and *S. officinalis.* Even higher levels of species discrimination power can be achieved by the use of a second marker region. The combined use of the two different plastidal marker regions rbcl and matK was proposed by the Consortium for the Barcode of Life (CBOL) plant working group as a standard barcode for land plants (Ausubel, 2009; Hollingsworth et al., 2009). These two regions were selected due to their universality and high levels of species discrimination, recoverability and sequence quality. It must, however be pointed out that this working group was aiming at plant phylogenetic studies, requiring marker regions with high phylogenetic resolution power. Here, the ITS region has some limitations, e.g. due to problems of paralogues in at least some groups of plants (Chase et al., 2005). Nonetheless, we chose the ITS region as barcode, as, in most groups of flowering plants, it has a similar performance compared to the plastidal markers, and in some cases, provides even higher levels of species discrimination (Hollingsworth et al., 2009). For our purposes, the ITS region provided sufficient power for discrimination of species. In addition, the starting material investigated by the plant working group mostly were the plant leaves, dried, but not further processed. Use of ITS is also supported by a large body of sequence data which is already available for this region in GenBank (Kress et al., 2005; Orland et al., 2014). The ITS region is not applicable for barcoding of all land plants, due to lacks of universality, but proved to be an

appropriate marker region for the reliable and efficient authentication of selected medicinal plants in the present work.

Table 42: Summary of the samples identified by ITS barcoding in dependence of processing procedures, number of extracts investigated.

	No. of samples investigated	Sequences obtained		Adulterants found		Complex processing of herbal substances		Ethanolic extracts of herbal substances	
		No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Herbal substances from the Lamiaceae family	33	33	100	3	9.1	-	-	-	-
Herbal substances from TCM	60	31	51.6	6	10	33	55	-	-
Cimicifugae racemosae rhizoma	36	30	83	5	13.9	-	-	3	8.3

5.2.2 Evaluation of DMHS with PCR-based approaches

Particular emphasis of the present work was placed on the PCR-based investigation of DMHS and finished medicinal products containing a mixture of herbal substances. For this purpose, three different PCR-based approaches were established and evaluated.

5.2.3 Investigation of DMHS with unknown content via cloning

The cloning approach was envisaged to complement the ITS barcoding method with the option to analyze herbal mixtures. It proved to be particularly appropriate as a preliminary tool to investigate mixtures with unknown content. Initial conclusions regarding the possible composition of a plant mixture may be drawn. However, this method is not exhaustive and results are limited by special amplification patterns of the *Taq* polymerase. In the test substance propolis it was a suitable approach to detect possible plant sources for propolis production, in line with data in literature. Additionally, results from microscopic analyses of propolis samples were available by a cooperation with Prof. Chinou from the University of Athens. It was thus possible to combine a conventional method for analysis of propolis samples with the cloning method. With both methods, species were detected that are reported as source for

propolis production. Accordingly, the approach has the potential to synergistically supplement microscopic analyses (see: 4.4.2 to 4.4.4.2) of propolis. Most sequencing results we obtained suited quite well to the plant species that were already referred to as origin for the production of propolis detected by conventional methods. Thus, it is conceivable that further research studies focusing on the plant origin of propolis samples could be complemented by this PCR-based approach. A similar method targeting algal chloroplast markers *rbcL* and *tufA* was used for retracing possible food sources of sacoglossan sea slugs by barcoding of sequestered chloroplasts (Christa et al., 2013, 2014). As another barcoding approach for the analysis of herbal mixtures with unknown contents, a pyrosequencing method was established by the working group of Taberlet aiming at the deciphering of the diet of small herbivores (Soininen et al., 2009; Valentini et al., 2009a).

To elaborate the efficiency of the method, also mixtures with known composition were analyzed. However, the investigation of a defined mixture with 5 components and the finished herbal medicinal products Sinupret® and Imupret® with this method yielded a low retraceability of components of the mixtures. Interestingly, the analysis of the DMHS with two different universal primer pairs yielded different results. With the primer pair TKA *M. recutita* and *Q. robur* were detected in the DMHS, while using the primer pair TKB *J. regia* and *M. recutita* were recovered out of the same mixture. Thus, the use of two different universal primers provided a higher detection rate of components of the defined herbal mixture.

Many steps in PCR analyses of mixtures are accessible to methodical errors. The method used for DNA extraction, the primer-design, PCR conditions as well as the procedure of cloning affect the results (Rainey et al., 1994). The relative frequency of genes in PCR products from mixed-template PCR reactions is influenced by many factors. First of all, unequal sampling or differences in the efficiency of the DNA isolation causes bias in the PCR reaction. Since, in case of a less effective DNA isolation of one component in a mixture, only few molecules are disposable to initiate the PCR amplification for this component (Suzuki and Giovannoni, 1996).

Basic problems of this approach are caused by inconsistencies regarding the amplification of templates of the *Taq* polymerase. A preferential amplification of templates with lower GC contents or of shorter templates is discussed and described in literature. The percentage of guanine and cytosine-content of template DNA and

primer has a major impact on the gene amplification by PCR (Dutton et al., 1993; Reysenbach et al., 1992; Suzuki and Giovannoni, 1996). High-GC-contents of a template result in a lower efficiency of dissociation into single-stranded molecules and by this, may promote the preferential amplification of templates with lower GC-contents (Suzuki and Giovannoni, 1996). The single stranded molecules are directly available for annealing with primers and cause disequilibrium in favor of the lower GC-containing templates. These effects may be circumvented by elevation of the denaturation temperature or the duration of the denaturation cycle (Walsh et al., 1992), or diminished by addition of co-solvents like acetamide (Reysenbach et al., 1992) or betaine (Weissensteiner and Lanchbury, 1996). Not least, the concentration of salt or co-solvents has to be adjusted carefully. Minor differences in the length of the ITS regions of different plants produce amplicons that may vary in length. In this case, a preferential amplification of shorter templates can occur (Walsh et al., 1992).

This may have led to incomplete and unequal recovery of plant components in known plant mixtures (finished medicinal products and a defined mixture of herbal substances: 4.5.1). The recovery rate did not significantly improve or change with growing numbers of samples analyzed, which became evident with the analysis of 50 colonies in case of the defined mixture (figure 35).

Due to the lack of completeness, this method is not suitable for a reliable and complete authentication of DMHS in terms of quality control. Notwithstanding, this method is, beneath the here tested PCR-based methods, the only approach applicable for at least partial detection of plants in mixtures with unknown content.

5.2.4 Investigation of DMHS with known content by multiplex PCR

In a multiplex PCR approach, specific primers were designed for the time-saving, simultaneous detection of five different components of a plant mixture. In a mixture, where all components were derived from single DNA isolations of each plant and then mixed, all components in equal concentrations were successfully detected (4.5.2; 4.5.2.1). In contrast, in mixtures of herbal substances, where DNA was isolated from the complete mixture, the detection of *A. clematitis* failed completely (4.5.2.2).

After the conception of specific primers, a theoretical PCR run provided by the used software, showed specificity of primers, when used for amplification of the inappropriate templates. But a practical specificity testing resulted in additional

amplification of unspecific components of the mixture. Hence, a disadvantage is a complex development process that includes a comprehensive specificity testing and the careful calibration of the appropriate primer concentration, enabling the parallel amplification of all components of the mixture.

Multiplex PCR systems were already established for the quality assessment of medicinal plants (Jigden 2010, Xue 2007b). Despite a complex establishment procedure it is a highly accurate and time-saving method for the identification of mixtures of herbal substances or finished medicinal products. However its application is limited by the visibility on the agarose gel, thereby providing a detection limit of 2-4 ng of conventional PCR; DNA concentrations below this limit cannot be detected anymore. This may be a limiting factor for the application of this method for complementary quality control of finished herbal medicinal products or processed drugs, where the efficiency of DNA isolation is low (Kersten, 2013).

5.2.5 Investigation of DMHS with known content via multiplexed qPCR

The multiplex qPCR-approach in combination with TaqMan® probes established in this project evolved to be an extremely sensitive method to detect even minor amounts of plant material in mixtures (see 4.5.4) and was a reliable approach to analyze the finished medicinal product Imupret® (see: 4.5.4.1). All plant components in the defined mixtures and the plant species *M. recutita*, *J. regia* and *Q. robur* that are also present in Imupret®, were detectable in both types of samples. It was thus a more effective method for analyzing mixtures than the conventional multiplex PCR approach.

In plant science, multiplex (TaqMan®) qPCR assays were so far established for the detection of plant viruses (Dai et al., 2013; Osman et al., 2013), or the detection of genetically modified organisms in crop plants/food industry (Cottenet et al., 2013). In terms of quality control of medicinal plants, high resolution melting analyses were already performed (Mader et al., 2011; Schmiderer et al., 2010). To our knowledge, the here established multiplex qPCR approach was first time used for the quality assessment of herbal substances and a finished herbal medicinal product.

The high sensitivity and specificity of qPCR assays requires a careful design of primers and probes, as well as calibrating appropriate primer concentrations of the competing

primer pairs and suitable $MgCl_2$ concentrations. Additionally, a laborious specificity testing (see 4.5.3.1) is mandatory to avoid cross-annealing of primers.

However, the benefits of high efficiency due to a very low detection limit of 2 to 20 pg DNA (Bustin, 2000; Mackay et al., 2002) and the high specificity enforced by the combined use of specific primers and probes outweigh these disadvantages and justify the complex establishment procedure. The low detection limit provided by this approach is especially promising for low yields of DNA from processed drugs and finished herbal medicinal products. Minor amounts of DNA and resulting inefficient amplification rates during PCR often pose a problem in conventional PCR applications.

5.2.6 Metabolic fingerprinting

A metabolomics approach, aiming at the fast and comprehensive high-throughput analysis of crude extracts, was established in a former PhD project (Daniel, 2009). In the present work, this method was further evaluated using medicinal plants from European and Asian therapeutic systems and propolis. Furthermore, a database, established by Daniel (2009) and complemented by (Orland et al., 2014) was supplemented with records of ^{1}H-NMR spectra of DCM extracts of herbal substances. By means of ^{1}H-NMR-fingerprinting in combination with PCA, the applicability and limitations of this approach for distinguishing plants belonging to the Lamiaceae at the genus and species level was evaluated.

A classification down to the species level was achieved using plants from the genus *Salvia* (4.1.2). The metabolomics approach has some limitations concerning the differentiation of plant species with a similar profile of secondary metabolites. Due to the usage of DCM as extraction solvent, mainly the lipophilic compounds, often found in species of the Lamiaceae, are extracted. In a PCA analysis comparing different genera of the Lamiaceae family, the genera *Salvia* and *Rosmarinum* were not successfully separated. Presumably as a result from similar essential oil components of *Rosmarinum officinalis*, *Salvia triloba* and *Salvia officinalis*, overlapping plots were obtained for these 3 species.

The ITS barcoding approach especially turned out to be a suitable method for exclusion or confirmation of adulterants found via metabolic fingerprinting. For exclusion or confirmation of adulterants found via the ITS barcoding approach especially the

combination of PCR-based approaches with metabolic fingerprinting turned out to be a suitable method. The adulterants *Acer rubrum* (Ci 10), *Morus murrayana* (Ci 11) and *Collinsonia canadensis* could theoretically be nearly excluded through a parallel analysis by metabolic fingerprinting, as even small admixtures were found to result in significant changes after application of the PCA analysis (Daniel, 2009). In case of another sample of Cimicifugae racemosae rhizoma (Ci 28), where the adulterant *Sonchus arvensis* was detected by PCR analyses, the sample indeed turned out to be an outlier in the PCA analysis (figure 22). Here, the identity of the sample has to be further evaluated with analytical methods, for a clear clarification of the origin of this sample.

Furthermore, a PCA of all *A. racemosa* samples revealed inconsistencies regarding three samples that were ordered via internet. They clustered clearly separated from samples with known origin that were all certified. Here, further analytical methods are required additionally to be able to characterize the chemical profile of the samples in question.

The analysis of propolis with the metabolomics approach, showed, that it is possible to retrace propolis capsules from different suppliers that presumably had an almost identical composition (4.4.1, figure 25). In general, propolis is a very variable product that can hardly be standardized (Bankova, 2005; Burdock, 1998; Salatino et al., 2005). Due to its diversity regarding the origin, very different samples may cluster because of a similar composition. This diversity limits the applicability of the metabolic fingerprinting approach, as small variations in the origin source can lead to significant varieties concerning a PCA analysis of these samples.

Depending on the choice of solvent used for the extractions, different classes of compounds will be extracted out of the plants metabolite spectrum. With DCM, mainly lipophilic compounds are extracted, which was an appropriate solvent for the extraction of essential oils of the Lamiaceae. However, quality control in line with pharmacopeial standards is mostly relying on marker substances. Hence, depending on extraction solvent used for the metabolic approach, the main active substances of medicinal plants may not be extracted.

However, a sample that was supposed to be *S. triloba*, based on a first macroscopic examination, clustered with samples of *S. officinalis* in a PCA analysis. By means of

further analytical and PCR-based methods this sample actually was identified as *S. officinalis*. Consequently, the PCA evaluation gave the hint for the misidentification.

For a reliable application of ^{1}H-NMR-fingerprinting for the complementary quality assessment of herbal substances it is mandatory to establish a database with a comprehensive, statistically significant number of valid reference samples. Also reference samples with different geographical origin should be taken into account to establish a reference database.

Recent studies indicated that ^{1}H-NMR-fingerprinting is rather not suitable for the reliable detection of plant adulterations in herbal substances or herbal medicinal products. Toxic substances do not necessarily contribute to the classification of samples and therefore may remain undetected (Orland et al., 2014). It is therefore of primary importance also to consider conventional methods for quality control of herbal substances described in the Ph. Eur..

5.3 Conclusion and Outlook: Investigation of herbal substances and their mixtures

Results from the present work indicate that complementary methods can efficiently sustain standard methods provided by pharmacopeias. Some of the methods tested are especially meeting the demands of challenging samples like mixtures of herbal substances or processed samples. Among the three different PCR-related approaches elaborated for the investigation of mixtures of herbal substances, especially the multiplexed qPCR approach seems suitable for a reliable and sensitive detection of plant species in mixtures. Owing to the high sensitivity provided by this approach, reliable results were also achieved concerning the investigation of finished herbal medicinal products. The application of this approach to analyse ethanolic extracts of herbal substances was not tested in the scope of this work, but is a promising tool to be tested in future prospects. Furthermore, the conception of a multiplexed qPCR assay would be conceivable, enabling the simultaneous detection of potentially toxic or allergenic plants, aiming at quality control of herbal substances.

In general, the comparative sequence analysis by barcoding proved to be a promising approach for the analysis of herbal substances. However, problems arising from all barcoding approaches investigated here may be strongly processed samples, ethanolic extracts of herbal substances, GC-rich sequences and the data underlying the sequence analyses. DNA-sequences used for the BLAST analyses are provided by an open access data base, containing entries that are not verified, and for some plants, no reference data is available. An important prerequisite for the application of barcoding for quality assessment of herbal substances is the realization of a comprehensive database with valid reference sequences. For the univocal authentication of closely related species the additional use of a second marker region could be essential, as proposed also by the CBOL working group (Ausubel, 2009; Hollingsworth, 2008). In order to broaden the scope of application to strongly processed drugs, amplification of ITS1 or ITS2 alone, or an alternative marker region may be tested, suitable for the amplification of degraded DNA. In general, DNA regions exceeding 150 bp may be difficult to be amplified from degraded DNA (Valentini et al., 2009b).

Metabolic fingerprinting using ^{1}H-NMR spectra combined with PCA analysis proved to be a useful method for an initial classification of samples, as well as for the detection of outliers. However, its application for samples investigated in this work was limited by similar metabolite spectra that may be extracted from different plant species and therefore may not be separated clearly. Due to the fact, that this approach is underlying a statistic analysis, the establishment of a comprehensive database, provided with valid reference samples, is indispensable.

In conclusion, pharmacopeial methods remain an indispensable tool for quality control of herbal substances but can be efficiently supplemented by complementary methods investigated in the present work. The strengths are to be found wherever conventional methods reach their limits or become extremely complex. Notwithstanding, further work is needed to address this issue and confirm validity and reliability for a broader range of applications in the field of quality assessment of herbal substances.

6 Summary

Herbal substances and preparations thereof play an important role in healthcare systems worldwide. An essential prerequisite for the safe and effective use of herbal medicines is the unequivocal identification of the plant species used therein. The majority of herbal substances are administered as multicomponent mixtures, especially in the field of TCM and ayurvedic medicine, but also in finished medicinal products used in Germany. Quality assessment of complex mixtures of herbal substances with conventional methods is challenging and time consuming. Thus, emphasis of the present work was directed on the development of complementary methods to elucidate the composition of mixtures of herbal substances and finished herbal medicinal products, focusing on options provided by PCR-related methods.

A multiplexed qPCR method in combination with TaqMan® probes has been established, enabling the reliable and precise detection of each species in a defined mixture of herbal substances composed of Aristolochiae herba, Quercus cortex, Juglandis folium, Matricariae flos and Salviae miltiorrhizae radix et rhizoma. This approach evolved to be highly sensitive and specific, and also suitable for minor DNA amounts yielded from processed plants or finished medicinal products. All components of the defined mixtures of herbal substances could be successfully detected in mixtures with highly variable quantities of each plant. The applicability of the method was also tested for the finished medicinal product Imupret®, which, amongst other herbal substances, also contains Quercus cortex, Juglandis folium and Matricariae flos. All expected components were detected, thereby achieving limits of detection of 20 pg DNA for the component Quercus cortex. Using a conventional multiplex PCR approach, the five components of the defined mixture of herbal substances could be readily verified, when equal DNA concentrations of all components were available, but failed in the complete detection of all plants in the application for mixtures with varying contents of each plant.

Due to recurrent concerns of contaminations of herbal substances with toxic plant species, the aristolochic acids - containing species *Aristolochia clematitis* (Aristolochiae herba) was part of the defined mixtures of herbal substances analyzed. This species was traceable by the qPCR approach in different defined mixtures of herbal substances up to an amount of 0.8 mg of herbal substance material of

Aristolochiae herba prior to DNA isolation, corresponding to only 4 % of the total amount of plant material in the mixture.

For the analysis of mixtures of herbal substances with unknown identity a PCR-based approach, including a cloning step and application of universal primers, turned out to be a valuable tool. This method was also applied to propolis, the composition of which is highly variable depending on the origin. A microscopic analysis of propolis provided additional information about plant species of specific samples. This result indicated that the combination of different methods may be very useful to support quality assessment.

An additional objective of the present thesis was further testing of special applications of an ITS barcoding approach (Kersten, 2013) and a ^{1}H-NMR fingerprinting technique in combination with multivariate data analysis (Daniel, 2009) for authentication purposes. The potential of the methods was evaluated using medicinal plants derived from the Lamiaceae family, Asian therapeutic systems (TCM herbal substances), the herbal substance Cimicifugae racemosae rhizoma and the plant-derived product propolis, thus supplementing already existing ITS-sequence and metabolomics data bases with valid reference data. The ITS barcoding approach proved to be a valid and quite robust method for the sensitive and unambiguous authentication of individual components of herbal substances. Special challenges were posed by processed material or herbal substances containing high amounts of essential oils. In total, 83 out of 129 samples of herbal substances belonging to the Lamiaceae family, TCM and Cimicifugae racemosae rhizoma were successfully authenticated by this method. However, the method encountered some problems regarding highly processed herbal substances, especially those from TCM, and ethanolic plant extracts.

In the present work, the ^{1}H-NMR fingerprinting approach was evaluated for its applicability to discriminate different plant genera and different plant species. For this application, there are limitations due to the presence of similar compounds occurring in different species or even different genera. It could be demonstrated that the application of this method should be restricted to a defined area and must be based on comprehensive, valid reference samples. As the evaluation with principal component analysis is a statistic method, the amount of reference samples should be significant, as the validity and relevance of the method improves with increasing number of reference samples. In case of Cimicifugae racemosae rhizoma, this method

gave a hint for commercially available samples that may not comply with pharmacopeial standards, but this assumption needs to be further evaluated with more sophisticated analytical methods.

Conventional analytical methods often have some limitations with respect to mixtures of herbal substances and finished herbal medicinal products. Minor amounts of sample material, processed herbal substances and complex mixtures place high demands on efficient quality control. Such prerequisites in many cases are hardly compatible with analytical methods that mostly are focusing on the detection of marker compounds. Results from the present work indicate that DNA-based methods can provide a suitable complementary approach for the sensitive and unambiguous authentication of herbal substances and their mixtures.

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8 Appendix

8.1 Herbal substances

Herbal substances investigated in the present work were purchased in pharmacies, taken from the gardens of the Institute of Pharmaceutical Biology or the gardens of useful plants in Bonn or provided by the EDQM. The plants taken from the gardens of the Institute of Pharmaceutical Biology or the gardens of useful plants in Bonn were identified by a botanist and dried at room temperature and air circulation prior to use for DNA isolations or analytical investigations.

Table 43: Herbal substances from TCM provided by the EDQM

Herbal substance	Pinyin	Species (Family)	EDQM code
Acanthopanacis cortex	Wu Jia Pi	*Acanthopanax gracilistylus W.W.Sm.* (Araliaceae)	32527
Acanthopanacis cortex	Wu Jia Pi	*Acanthopanax gracilistylus W.W.Sm.* (Araliaceae)	32961
Acanthopanax giraldii caulis/cortex		*Acanthopanax giraldii* Harms (Araliaceae)	32987
Aconiti kusnezoffii radix preparata	Zhi Cao Wu	*Aconitum kusnezoffii* Rchb. (Ranunculaceae)	33022
Aconiti radix praeparata	Zhi Chuan Wu	*Aconitum carmichaeli* Debeaux (Ranunculaceae)	32969
Aconiti radix praeparata	Zhi Chuan Wu	*Aconitum carmichaeli* Debeaux (Ranunculaceae)	29242
Aconiti radix lateralis praeparata	Fu Zi	*Aconitum carmichaeli* Debeaux (Ranunculaceae)	32994
Aconiti radix lateralis praeparata	Fu Zi	*Aconitum carmichaeli* Debeaux (Ranunculaceae)	33032
Acori calami	Shui Chang Pu	*Acorus calamus* L. (Araceae)	33000
Acori tatarinowii rhizoma	Shi Chang Pu	*Acorus tatarinowii* Schott (Araceae)	32976
Acori tatarinowii rhizoma	Shi Chang Pu	*Acorus tatarinowii* Schott (Araceae)	32515
Acori tatarinowii rhizoma authentic	Shi Chang Pu	*Acorus tatarinowii* Schott (Araceae)	31403
Anemarrhenae rhizoma	Zhi Mu	*Anemarrhena asphodeloides* Bunge (Liliaceae)	32964
Anemarrhenae rhizoma	Zhi Mu	*Anemarrhena asphodeloides* Bunge (Liliaceae)	32483
Anemarrhenae rhizoma praeparata	Yan Zhi Mu	*Anemarrhena asphodeloides* Bunge (Liliaceae)	32988

Herbal substance	Pinyin	Species (Family)	EDQM code
Arnebiae radix	Zi Cao	*Arnebia euchroma; A. guttata* Bunge; *Lithospermum erythrorhizon* (Siebold & Zuccarini) Handel-Mazzetti (Boraginaceae)	32530
Arnebiae radix	Zi Cao	*Arnebia euchroma*; *A. guttata; Lithospermum erythrorhizon* (Siebold & Zuccarini) Handel-Mazzetti (Boraginaceae)	32979
Arnebiae radix authenthic	Zi Cao	*Arnebia euchroma; A. guttata; Lithospermum erythrorhizon* (Siebold & Zuccarini) Handel-Mazzetti (Boraginaceae)	31399
Asari radix et rhizoma	Xi Xin	*Asarum heterotropoides var. mandshuricum* Maximowicz; *A.sieboldii var. seoulense* Nakai*; A. sieboldii* Miq. (Aristolochiaceae)	32540
Astragali radix authentic	Huang Qi	*Astragalus membranaceus* Bge. *var. mongholicus* (Bge.); *A. membranaceus* Bge.(Leguminosae)	31413
Astragali radix	Huang Qi	*Astragalus membranaceus* Bge. *var. mongholicus* (Bge.); *A. membranaceus* Bge.(Leguminosae)	32487
Astragali radix	Huang Qi	*Astragalus membranaceus* Bge. *var. mongholicus* (Bge.); *A. membranaceus* Bge.(Leguminosae)	32999
Aucklandiae radix	Mu Xiang	*Aucklandia lappa* Decne. (Compositae)	29254
Aucklandiae radix	Mu Xiang	*Aucklandia lappa* Decne. (Compositae)	33027
Belamcandae rhizoma	She Gan	*Belamcanda chinensis* (L.) DC. (Iridiaceae)	32488
Belamcandae rhizoma	She Gan	*Belamcanda chinensis* (L.) DC. (Iridiaceae)	32967
Bupleuri radix	Chai Hu	*Bupleurum chinense* DC.*, B. scorzonerifolium* Willd. (Umbelliferaceae)	32537
Bupleuri radix	Chai Hu	*Bupleurum chinense* DC.*, B. scorzonerifolium* Willd. (Umbelliferaceae)	32974
Bupleuri rhizoma		*Bupleurum chinense* DC.*, B. scorzonerifolium* Willd. (Umbelliferaceae)	33017

Herbal substance	Pinyin	Species (Family)	EDQM code
Chaenomelis fructus	Mu Gua	*Chaenomeles speciosa* (Sweet) Nakai (Rosaceae)	32533
Chaenomelis fructus	Mu Gua	*Chaenomeles speciosa* (Sweet) Nakai (Rosaceae)	33051
Citri reticulatae pericarpium	Chen Pi	*Citrus reticulata* Blanco (Rutaceae)	29265
Citri reticulatae pericarpium	Chen Pi	*Citrus reticulata* Blanco (Rutaceae)	33021
Citri reticulatae pericarpium authentic	Chen Pi	*Citrus reticulata* Blanco (Rutaceae)	31408
Clematidis armandii caulis	Chuan Mu Tong	*Clematis armandii* Franch., *C. montana* Buch.- Ham. (Ranunculaceae)	33055
Clematidis armandii		*Clematis armandii* Franch., *C. montana* Buch.- Ham. (Ranunculaceae)	29233
Coicis semen	Yi Yi Ren	*Coix lacryma -jobi var. ma-yuen* (Roman.) Stapf (Gramineae)	29251
Coicis semen	Yi Yi Ren	*Coix lacryma -jobi var. ma-yuen* (Roman.) Stapf (Gramineae)	32528
Coicis semen	Yi Yi Ren	*Coix lacryma -jobi var. ma-yuen* (Roman.) Stapf (Gramineae)	33010
Coptidis rhizoma	Huang Lian	*Coptis chinensis* Franch., *C. deltoides* C.Y. Cheng & Hsiao, *C. teeta* Wall. (Ranunculaceae)	32531
Coptidis rhizoma	Huang Lian	*Coptis chinensis* Franch., *C. deltoides* C.Y. Cheng & Hsiao, *C. teeta* Wall. (Ranunculaceae)	32957
Daturae flos		*Datura stramonium var. tatula* (L.) Torr. (Solanaceae)	33002
Dioscoreae rhizoma	Shan Yao	*Dioscorea opposita* Thunb. (Dioscoreaceae)	32497
Dioscoreae rhizoma	Shan Yao	*Dioscorea opposita* Thunb. (Dioscoreaceae)	33052
Dioscoreae rhizoma praeparata	Guang Shan Yao	*Dioscorea opposita* Thunb. (Dioscoreaceae)	32991
Drynariae rhizoma	Gu Sui Bu	*Drynaria fortunei* (Kunze) J. Sm (Polypodiaceae)	32943
Ecliptae herba	Mo Han Lian	*Eclipta prostrata* L.(Asteraceae)	32520
Ecliptae herba	Mo Han Lian	Eclipta prostrata L. (Asteraceae)	32971
Ephedrae herba	Ma Huang	*Ephedra sinica* Stapf, *E. intermedia* Schrenk & C.A.Mey., *E. equisetina* Bge. (Ephedraceae)	32940

Herbal substance	Pinyin	Species (Family)	EDQM code
Ephedrae herba praeparata	Zhi Ma Huang	*Ephedra sinica* Stapf, *E. intermedia* Schrenk & C.A.Mey., *E. equisetina* Bge. (Ephedraceae)	32939
Eucommiae cortex praeparata	Chao Du Zhong	*Eucommia ulmoides* Oliv. (Eucommiaceae)	32975
Eupatorii herba	Pei Lan	*Eupatorium fortunei* Turczaninow (Compositae)	32499
Eupatorii herba	Pei Lan	*Eupatorium fortunei* Turczaninow (Compositae)	32985
Evodiae fructus	Wu Zhu Yu	*Evodia rutaecarpa* (Juss.) Benth., *E. rutaecarpa* (Juss.) Benth. var. *officinalis* (Dode) Huang, *E. rutaecarpa* (Juss.) Benth. var. *bodinieri* (Dode) Huang (Rutaceae)	33001
Farfare flos	Kuan Dong Hua	*Tussilago farfara* L. (Compositae)	32518
Farfare flos	Kuan Dong Hua	*Tussilago farfara* L. (Compositae)	33007
Farfare flos	Kuan Dong Hua	*Tussilago farfara* L. (Compositae)	33043
Forsythiae fructus	Lian Qiao	*Forsythia suspensa* (Thunb.) Vahl (Oleaceae)	32529
Forsythiae fructus	Lian Qiao	*Forsythia suspensa* (Thunb.) Vahl (Oleaceae)	32978
Gardeniae fructus	Zhi Zi	*Gardenia jasminoides* Ellis (Rubiaceae)	32496
Gardeniae fructus	Zhi Zi	*Gardenia jasminoides* Ellis (Rubiaceae)	33031
Gardeniae fructus praeparata	Chao Zhi Zi Jiao Zhi Zi	*Gardenia jasminoides* Ellis (Rubiaceae)	32990
Gardeniae fructus praeparata	Chao Zhi Zi Jiao Zhi Zi	*Gardenia jasminoides* Ellis (Rubiaceae)	33024
Gastrodiae fhizoma	Tian Ma	*Gastrodia elata* Bl. (Orchidaceae)	33029
Houttuyniae herba	Yu Xing Cao	*Houttuynia cordata* Thunb. (Saururaceae)	25846
Houttuyniae herba	Yu Xing Cao	*Houttuynia cordata* Thunb. (Saururaceae)	32484
Houttuyniae herba	Yu Xing Cao	*Houttuynia cordata* Thunb. (Saururaceae)	32984
Indigo naturalis	Qing Dai	*Baphicacanthus cusia* (Nees) (Verbenaceae); *Polygonum tinctorium* Ait (Polygonaceae); *Isatis indigotica* Fort. (Cruciferae)	33033

Herbal substance	Pinyin	Species (Family)	EDQM code
Indigo naturalis authentic	Qing Dai	*Baphicacanthus cusia* (Nees) (Verbenaceae); *Polygonum tinctorium* Ait (Polygonaceae); *Isatis indigotica* Fort. (Cruciferae)	31410
Indigo naturalis	Qing Dai	*Baphicacanthus cusia* (Nees) (Verbenaceae); *Polygonum tinctorium* Ait (Polygonaceae); *Isatis indigotica* Fort. (Cruciferae)	31498
Isatidis radix	Ban Lan Gen	*Isatis indigotica* Fort. (Cruciferae)	25847
Isatidis radix	Ban Lan Gen	*Isatis indigotica* Fort. (Cruciferae)	32498
Isatidis radix	Ban Lan Gen	*Isatis indigotica* Fort. (Cruciferae)	33048
Ligustici rhizoma et radix	Gao Ben	*Ligusticum sinense* Oliv.; *L. jeholense* Nakai et Kitag. (Umbelliferae)	32492
Ligustici rhizoma et radix	Gao Ben	*Ligusticum sinense* Oliv.; *L. jeholense* Nakai et Kitag. (Umbelliferae)	32938
Ligustici rhizoma et radix authentic	Gao Ben	*Ligusticum sinense* Oliv.; *L. jeholense* Nakai et Kitag. (Umbelliferae)	31409
Lycii fructus	Gou Qi Zi	*Lycium barbarum* L. (Solanaceae)	32494
Magnoliae officinalis cortex	Huo Po	*Magnolia officinalis* Rehd. Et Wils; *M. officinalis var. Biloba* (Magnoliaceae)	32502
Magnoliae officinalis cortex	Xin Yin	*Magnolia officinalis* Rehd. Et Wils; *M. officinalis var. Biloba* (Magnoliaceae)	32949
Magnoliae officinalis flos	Xin Yin	*Magnolia officinalis* Rehd. Et Wils; *M. officinalis var. Biloba* (Magnoliaceae)	32944
Notopterygii rhizoma et radix	Qiang Huo	*Notopterygium incisum* Tind ex H.T. Chang, *N. forbesii* Boiss. (Apiaceae)	32525
Notopterygii rhizoma et radix	Qiang Huo	*Notopterygium incisum* Tind ex H.T. Chang, *N. forbesii* Boiss. (Apiaceae)	32981
Pinelliae praeparatum rhizoma	Fa Ban Xia	*Pinelliae ternata* syn. *Arum ternatum* Thunb. (Araceae)	32516
Pinelliae praeparatum rhizoma	Fa Ban Xia	*Pinelliae ternata* syn. *Arum ternatum* Thunb. (Araceae)	33013
Pinelliae praeparatum rhizoma	Fa Ban Xia	*Pinelliae ternata* syn. *Arum ternatum* Thunb. (Araceae)	33009

Herbal substance	Pinyin	Species (Family)	EDQM code
Pinelliae rhizoma	Ban Xia	*Pinelliae ternata* syn. *Arum ternatum* Thunb. (Araceae)	33046
Poria	Fu Ling	*Poria cocos* (Schw.) Wolf. (Polyporaceae)	32505
Poria	Fu Ling	*Poria cocos* (Schw.) Wolf. (Polyporaceae)	32992
Poria	Fu Ling	*Poria cocos* (Schw.) Wolf. (Polyporaceae)	32983
Puerariae lobatae radix	Ge Gen	*Pueraria lobata* (Willd.) (Leguminosae)	29245
Puerariae lobatae radix	Ge Gen	*Pueraria lobata* (Willd.) (Leguminosae)	32504
Puerariae lobatae radix	Ge Gen	*Pueraria lobata* (Willd.) (Leguminosae)	33059
Rhei radix et rhizoma	Da Huang	*Rheum palmatum* L., *R. tanguticum* Maxim. ex Balf., *R. officinale* Baill. (Polygonaceae)	33070
Rhei radix et rhizoma	Da Huang	*Rheum palmatum* L., *R. tanguticum* Maxim. ex Balf., *R. officinale* Baill. (Polygonaceae)	33071
Rhei radix et rhizoma	Da Huang	*Rheum palmatum* L., *R. tanguticum* Maxim. ex Balf., *R. officinale* Baill. (Polygonaceae)	33058
Salviae miltiorrhizae radix et rhizoma	Dan Shen	*Salvia miltorrhiza* Bge. (Lamiaceae)	33040
Salviae miltiorrhizae radix et rhizoma	Dan Shen	*Salvia miltorrhiza* Bge. (Lamiaceae)	32482
Saposhnikoviae radix	Fang Feng	*Saposhnikovia divaricata* (Turcz.) Schischk (Umbelliferae)	32490
Saposhnikoviae radix	Fang Feng	*Saposhnikovia divaricata* (Turcz.) Schischk (Umbelliferae)	33030
Schisandrae chinensis fructus	Wu Wei Zi	*Schisandra chinensis* (Turcz.) Baill. (Schisandraceae)	32514
Schisandrae chinensis fructus	Wu Wei Zi	*Schisandra chinensis* (Turcz.) Baill. (Schisandraceae)	33054
Scutellariae baicalensis georgi radix	Huang Qin	*Scutellaria baicalensis* Georgi (Lamiaceae)	30008
Scutellariae viscidulae radix	Huang Qin	*Scutellaria baicalensis* Georgi (Lamiaceae)	30009
Scutellariae radix	Huang Qin	*Scutellaria baicalensis* Georgi (Lamiaceae)	33025

Herbal substance	Pinyin	Species (Family)	EDQM code
Sinomenii caulis	Qing Feng Teng	*Sinomenium acutum* Diels oder *Sinomenium acutum var. cinereum* (Diels) Rehder & E.H. Wilson (Menispermaceae)	32982
Spica prunellae	Xia Ku Cao	*Prunella vulgaris* L. (Lamiaceae)	32524
Spica prunellae	Xia Ku Cao	*Prunella vulgaris* L. (Lamiaceae)	32986
Stephaniae tetrandrae radix	Fang Ji	*Stephania tetrandra* S. Moore (Menispermaceae)	29239
Stephaniae tetrandrae radix	Fang Ji	*Stephania tetrandra* S. Moore (Menispermaceae)	31412
Stephaniae tetrandrae radix authentic	Fang Ji	*Stephania tetrandra* S. Moore (Menispermaceae)	33020
Uncariae ramulus cum uncis	Gou Teng	*Uncaria rhynchophylla* (Miq.) Jacks., *U. macrophylla* Wall., *U. hirsuta* Havil., *U. sinensis* (Oliv.) Havil., *U. sessilifrucus* Roxb. (Rubiaceae)	32501
Zanthoxyli pericarpium	Hua Jiao	*Zanthoxylum schinifolium* Sieb. & Zucc.; *Z. bungeanum* Maxim. (Rutaceae)	32526
Zanthoxyli pericarpium	Hua Jiao	*Zanthoxylum schinifolium* Sieb. & Zucc.; *Z. bungeanum* Maxim. (Rutaceae)	32952
Ziziphi spinosae semen	Suan Zao Ren	*Ziziphus spinosa* Hu (Rhamnaceae)	32509
Ziziphi spinosae semen praeparata	Chao Suan Zao Ren	*Ziziphus spinosa* Hu (Rhamnaceae)	33011

Table 44: Herbal substances from TCM from pharmacies in Germany and from China.

Herbal substance (code)	Pinyin	Species (Family)	origin
Acori rhizoma (TCM 001)	Shi Chang Pu	*Acorus tatarinowii* Schott (Araceae)	Pharmacy Kaiser
Notoptergii rhizoma (TCM 002)	Qiang Huo	*Notopterygium incisum* Tind ex H.T. Chang, *N. forbesii* Boiss. (Apiaceae)	Pharmacy Kaiser
Coicis semen (TCM 003)	Yi Yi Ren	*Coix lacryma -jobi var. ma-yuen* (Roman.) Stapf (Gramineae)	Pharmacy Kaiser
Acori rhizoma granules (TCM 004)		*Acorus tatarinowii* Schott (Araceae)	Pharmacy Kaiser
Notoptergii rhizoma granules (TCM 005)	Qiang Huo	*Notopterygium incisum* Tind ex H.T. Chang, *N. forbesii* Boiss. (Apiaceae)	Pharmacy Kaiser

Herbal substance (code)	Pinyin	Species (Family)	origin
Salvia miltiorrhiza (TCM 009)	Dan Shen	*Salvia miltiorrhiza* Bge. (Lamiaceae)	China 2012
Salviae miltiorrhizae radix et rhizoma (TCM 010)	Dan Shen	*Salvia miltiorrhiza* Bge. (Lamiaceae)	China 2012
Salviae miltiorrhizae radix et rhizoma danshen Sichuan (TCM 011)	Dan Shen	*Salvia miltiorrhiza* Bge. (Lamiaceae)	China 2012
Danshen (TCM 012)	Dan Shen	*Salvia miltiorrhiza* Bge. (Lamiaceae)	China 2012
Salviae miltiorrhizae radix et rhizoma granules (TCM 013)	Dan Shen	*Salvia miltiorrhiza* Bge. (Lamiaceae)	Pharmacy Kaiser, Bonn

Table 45: Herbal substances, mostly of european origin

No.	Herbal substance	Species (Family)	Origin
1	Salviae miltiorrhizae herba (TD 002)	*Salvia miltiorrhiza* Bge. (Lamiaceae)	Pharmacy Kaiser, Bonn
2	Thymii herba (TD 003)	*Thymus vulgaris* L. (Lamiaceae)	Pharmacy Kaiser, Bonn
3	Salviae officinalis folium (TD 004)	*Salvia officinalis* L. (Lamiaceae)	Pharmacy Flora, Bonn
4	Salviae officinalis folium (TD 005)	*Salvia officinalis* L. (Lamiaceae)	Pharmacy Kaiser, Bonn
5	Salviae officinalis folium (TD006)	*Salvia officinalis* L. (Lamiaceae)	Pharmacy Kaiser, Bonn
6	Menthae piperitae folium (TD 007)	*Mentha* x *piperita* L. (Lamiaceae)	Pharmacy Kaiser, Bonn
7	Marrubbii herba(TD 009)	*Marrubium vulgare* L. (Lamiaceae)	Pharmacy Flora, Bonn
8	Althae officinalis radix (TD 012)	*Althaea officinalis* L. (Malvaceae)	Pharmacy Flora, Bonn
9	Juglandis folium (TD 013)	*Juglans regia* L. (Juglandaceae)	Pharmacy Flora, Bonn
10	Quercus cortex (TD 014)	*Quercus robur* L. (Fagaceae)	Pharmacy Flora, Bonn
11	Equiseti herba (TD 015)	*Equisetum arvense* L. (Equisetaceae)	Pharmacy Flora, Bonn
12	Matricariae flos (TD 016)	*Matricaria recutita* L. (Asteraceae)	Pharmacy Flora, Bonn
13	Juglandis folium (TD 018)	*Juglans regia* L. (Juglandaceae)	Praktikum Bio 2
14	Quercus cortex (TD 029)	*Quercus robur* L. (Fagaceae)	Pharmacy Hohenzollernapotheke Cologne
15	Coptidis rhizoma (TD 033)	*Coptis chinensis* Franch., *C. deltoides* C.Y. Cheng & Hsiao,	Pharmacy Flora, Bonn

No.	Herbal substance	Species (Family)	Origin
		C. teeta Wall. (Ranunculaceae)	
16	Salviae trilobae folium (TD 034)	*Salvia triloba* L. (Lamiaceae)	Praktikum Bio 2
17	Salviae miltiorrhizae rhizoma (TD 036)	*Salvia miltiorrhiza* Bge. (Lamiaceae)	Pharmacy Kaiser, Bonn
18	Menthae herba (TD 037)	*Mentha piperita L.* (Lamiaceae)	Pharmacy Kaiser, Bonn
19	Thymii herba (TD 038)	*Thymus vulgaris* L. (Lamiaceae)	Pharmacy Kaiser, Bonn
20	Menthae piperitae herba (TD 039)	*Mentha piperita* L. (Lamiaceae)	Pharmacy Kaiser, Bonn
21	Dioscoreae oppositae rhizoma (TD 043)	*Dioscorea opposita* Thunb. (Dioscoraceae)	Pharmacy Flora, Bonn
22	Salviae officinalis folium (TD 046)	*Salvia officinalis* L. (Lamiaceae)	Pharmacy Kaiser
23	Salviae officinalis folium (TD 047)	*Salvia officinalis* L. (Lamiaceae)	Pharmacy Flora, Bonn
24	Salviae officinalis folium (TD 048)	*Salvia officinalis* L. (Lamiaceae)	Pharmacy Münsterplatz, Bonn
25	Salviae officinalis folium (TD 049)	*Salvia officinalis* L. (Lamiaceae)	Pharmacy Rosen, Bonn
26	Salviae officinalis folium (TD 050)	*Salvia officinalis* L. (Lamiaceae)	Pharmacy Viktoria, Cologne
27	Salviae trilobae folium (TD 051)	*Salvia triloba* L. (Lamiaceae)	Galke
28	Salviae officinalis folium Charge 21103057 (TD 052)	*Salvia officinalis* L. (Lamiaceae)	PhytoLab
29	Salviae officinalis folium Charge 21103016 (TD 053)	*Salvia officinalis* L. (Lamiaceae)	PhytoLab
30	Salviae officinalis folium Charge 21101033 (TD 054)	*Salvia officinalis* L. (Lamiaceae)	PhytoLab
31	Salviae officinalis folium Charge 22100088 (TD 055)	*Salvia officinalis* L. (Lamiaceae)	PhytoLab
32	Salviae officinalis folium Charge 21106375 (TD 056)	*Salvia officinalis* L. (Lamiaceae)	PhytoLab
33	Salvia lavandulifolia Charge 20100345 (TD 057)	*Salvia lavandulifolia* Vahl. (Lamiaceae)	PhytoLab
34	Salviae trilobae folium Charge 21105963 (TD 058)	*Salvia triloba* L. (Lamiaceae)	PhytoLab
35	Verbasci flos (TD 059)	*Verbascum* sp. (Scrophulariaceae)	Pharmacy Flora, Bonn
36	Calendula flos (TD 060)	*Calendula officinalis* L. (Asteraceae)	Pharmacy Flora, Bonn
37	Thymii herba (TD 061)	*Thymus vulgaris* L. (Lamiaceae)	Pharmacy Flora, Bonn
38	Foeniculi fructus (TD 062)	*Foeniculum vulgare* Miller ssp. (Apiaceae)	Pharmacy Flora, Bonn

No.	Herbal substance	Species (Family)	Origin
39	Farfarae folium (TD 063)	*Tussilago farfara* L. (Asteraceae)	Pharmacy Flora, Bonn
40	Anserinae herba (TD 064)	*Potentilla anserina* L. (Rosaceae)	Pharmacy Flora, Bonn
41	Plantaginis folium (TD 065)	*Plantago lanceolata* L. (Plantaginaceae)	Pharmacy Flora, Bonn

Table 46: Herbal substances originating from the gardens of the Institute of Pharmaceutical Biology (all belonging to the family Lamiaceae) or the gardens of useful plants University Bonn

No.	Herbal substance	Species
1	Marrubbii herba (IG 001)	*Marrubium vulgare* L.
2	Salviae trilobae folium (IG 002)	*Salvia triloba* L.
3	Menthae crispae folium (IG 003)	*Mentha spicata* L. var*. crispa*
4	Menthae piperitae folium (IG 004)	*Mentha piperita* L.
5	Salviae officinalis folium (IG 005)	*Salvia officinalis* L.
6	Thymii herba (IG 006)	*Thymus vulgaris ssp. citriodorus*
7	Salviae divinorum folium (IG 007)	*Salvia divinorum* Epling & Jativa
8	Salviae officinalis folium (IG 008)	*Salvia officinalis* L.
9	Thymii herba (IG 009)	*Thymus vulgaris ssp. citriodorus*
10	Marrubbii herba (IG 010)	*Marrubium vulgare* L.
11	Menthae crispae folium (IG 011)	*Mentha spicata* L. var*. crispa*
12	Thymii herba (IG 012)	*Thymus vulgaris* L.
13	Salviae divinorum folium (IG 013)	*Salvia divinorum* Epling & Jativa
14	Salviae divinorum folium (IG 014)	*Salvia divinorum* Epling & Jativa
15	Salviae divinorum folium (IG 015)	*Salvia divinorum* Epling & Jativa
16	Salviae divinorum folium (IG 016)	*Salvia divinorum* Epling & Jativa
17	Salviae officinalis folium (IG 018)	*Salvia divinorum* Epling & Jativa
18	Salviae officinalis folium (IG 019)	*Salvia divinorum* Epling & Jativa
19	Salviae trilobae folium (IG 020)	*Salvia triloba* L. GUP (gardens of useful plants, Bonn)
20	Salviae pratensis folium (IG 021)	*Salvia pratensis* L.
21	Salviae pratensis folium (IG 021)	*Salvia pratensis* L.
22	Salviae pratensis folium (IG 021)	*Salvia pratensis* L.
23	Salviae pratensis folium (IG 021)	*Salvia pratensis* L.
24	Salviae splendens folium (IG 025)	*Salvia splendens* Sellow ex Roem. & Schult.
25	Salviae splendens folium (IG 026)	*Salvia splendens* Sellow ex Roem. & Schult.
26	Salviae splendens folium (IG 027)	*Salvia splendens* Sellow ex Roem. & Schult.
27	Salviae splendens folium (IG 028)	*Salvia splendens* Sellow ex Roem. & Schult.
28	Menthae longifoliae folium (IG 037)	*Mentha longifolia* (L.) Huds.
29	Rosmarini folium (IG 041)	*Rosmarinus officinalis* L.
30	Rosmarini folium (IG 042)	*Rosmarinus officinalis* L.

No.	Herbal substance	Species
31	Rosmarini folium (IG 043)	*Rosmarinus officinalis* L.
32	Rosmarini folium (IG 044)	*Rosmarinus officinalis* L.
33	Salviae officinalis folium (IG 049)	*Salvia officinalis* L. GUP (gardens of useful plants, Bonn)
34	Salviae trilobae folium (IG 020)	*Salvia triloba* L. GUP (garden of useful plants, Bonn)

Table 47: Finished medicinal products

No.	Finished medicinal product (FMP) / (code)	Contents	Origin
1	Sinupret® (FMP 001)	Primulae flos, Gentianae radix, Rumicis herba, Sambuci flos, Verbenae herba	Pharmacy in Bonn
2	Imupret® (FMP 002)	Althaea radix, Matricariae flos, Equiseti herba, Juglandis folium, Millefolii herba, Taraxaci herba,Quercus cortex	Pharmacy in Bonn

Table 48: Cimicifugae racemosae rhizoma samples (*Actaea racemosa*, Syn. *Cimicifuga racemosa* (L.) Nutt., Ranunculaceae)

No.	Sample	Origin
Ci 001	Nature´s Bounty Black Cohosh	Online Shop
Ci 002	Good´N Natural Black Cohosh	Online Shop
Ci 003	Herb Tech Black Cohosh	Online Shop
Ci 004	Eclectic Institute Motherwort Black Cohosh	Online Shop
Ci 005	Cimicifugae racemosae rhizoma conc.	Flora Apotheke
Ci 006	Black Cohosh-rhizoma, Cimicifugae Race 20678	EDQM
Ci 007	Black Cohosh-rhizoma, Cimicifugae Race 20679	EDQM
Ci 008	Cimicifugae racemosae rhizoma 26953	EDQM
Ci 009	Cimicifugae racemosae rhizoma 26954	EDQM
Ci 010	Schlangenwurzel schwarz 17801	PhytoLab
Ci 011	Schlangenwurzel schwarz 17802	PhytoLab
Ci 012	Schlangenwurzel schwarz 17803	PhytoLab
Ci 013	Schlangenwurzel schwarz 17804	PhytoLab
Ci 014	Schlangenwurzel schwarz 17805	PhytoLab
Ci 015	Cimicifugae rhizoma tot. ChargeNr. 36699	Bionorica AG
Ci 016	Cimicifugae rhizoma conc. ChargeNr. 26867	Bionorica AG
Ci 017	Cimicifugae rhizoma conc. ChargeNr. 27838	Bionorica AG
Ci 018	Cimicifugae rhizoma ChargeNr. 43321	Bionorica AG
Ci 019	Cimicifugae rhizoma ChargeNr. 43322	Bionorica AG

No.	Sample	Origin
Ci 020	Cimicifugae rhizoma ChargeNr. 43401	Bionorica AG
Ci 021	Cimicifugae rhizoma tot. ChargeNr. 38132	Bionorica AG
Ci 022	Cimicifugae rhizoma tot. ChargeNr. 38133	Bionorica AG
Ci 023	Cimicifugae rhizoma tot. ChargeNr. 35688	Bionorica AG
Ci 024	Cimicifugae rhizoma tot. ChargeNr. 38135	Bionorica AG
Ci 025	Cimicifugea rhizoma tot. ChargeNr. 38134	Bionorica AG
Ci 026	Cimicifugae rhizoma conc. ChargeNr. 14017	Bionorica AG
Ci 027	Starwest Botanicals Black Cohosh Root Powder	Online Shop (Wildcrafted)
Ci 028	Starwest Botanicals Black Cohosh Root Powder Organic	Online Shop (Wildcrafted)

8.2 Propolis samples

Propolis samples were kindly provided by Dete Papendiek from the Institute for Apiology of the University Bonn, by Prof. Chinou from the University of Athens or purchased via online-shops.

Table 49: Propolis samples (Crude Propolis, processed Propolis, Propolis extract)

No.	Sample (intern no.)	Origin
1	Propolis powder (PR 001)	Internetshop "Naturprodukte MV"
2	Propolis extract (PR 002)	Pharmacy CAELO
3	Crude propolis (PR 003)	Institute for Apiology, University Bonn
4	Crude propolis (PR 004)	Institute for Apiology, University Bonn
5	Crude propolis (PR 005)	Institute for Apiology, University Bonn
6	Green propolis powder Brazil (PR 006)	Internetshop "Der grüne Propolisshop"
7	Raw green propolis powder, capsules (PR 007)	Internetshop "The wild bee"
8	Pure propolis (PR 008)	Internetshop "Imkerei Robert Feuerstein"
9	Elusanes propolis, capsules (PR 009)	Pharmacy France
10	Propolis pure, capsules (PR 010)	"Peter´s Bestes", Pharmacy Austria
11	Propolis Zirkulin, capsules (PR 011)	Drug store, Germany
12	Crude propolis (PR 012)	Institute for Apiology, University Bonn
13	Crude propolis (PR 013)	Institute for Apiology, University Bonn
14	Crude propolis (PR 014)	Institute for Apiology, University Bonn
15	Crude propolis(PR 015)	Institute for Apiology, University Bonn
16	Crude propolis (PR 016)	Institute for Apiology, University Bonn
17	Aloe propolis Creme, Judy C. Sarkalla (PR 017)	Pharmcy, Germany
18	Bee Propolis, capsules (PR 018)	Pharmacy England, Holland & Barrett
19	Nature´s Defender Propolis,capsules (PR 019)	Pharmacy England, Bee Health
20	Propolis tincture (PR 020)	Internetshop "Imkerei Robert Feuerstein"
21	Propolis from grid 2010 Nr.1 (PR 021)	Institute for Apiology, University Bonn

No.	Sample (intern no.)	Origin
22	(Propolis tincture PR 022)	Institute for Apiology, University Bonn
23	Fruit-meadow 21.07.2011 (PR 036)	Institute for Apiology, University Bonn
24	Fruit-meadow 21.07.2011 (PR 037)	Institute for Apiology, University Bonn
25	„Kottenforst St. 7 Oberträger Folie 15.06.2011“ (PR 046)	Institute for Apiology, University Bonn
26	„Kottenforst St. 5 Oberträger Folie 15.06.2011“ (PR 047)	Institute for Apiology, University Bonn
27	„Winkelsweg Oberträger Ableger Folie 28.06.2011“ (PR 048)	Institute for Apiology, University Bonn
28	„Bee colony Park Oberträger Melb 08.06.2011“ (PR 049)	Institute for Apiology, University Bonn
29	Propolis Uruguay (crude) (PR 050)	Apiary Uruguay
30	Propolis Kirgisistan (crude) (PR 051)	Kirgisistan
31	Raw propolis Greece sample 1 (PR 052)	School of Pharmacy, University of Athens
32	Raw propolis Greece sample 2 (PR 053)	School of Pharmacy, University of Athens
33	Raw propolis Greece sample 3 (PR 054)	School of Pharmacy, University of Athens
34	Raw propolis Greece sample 4 (PR 055)	School of Pharmacy, University of Athens
35	Raw propolis Greece sample 5 (PR 056)	School of Pharmacy, University of Athens
36	Raw propolis Greece sample 6 (PR 057)	School of Pharmacy, University of Athens
37	Raw propolis Greece sample 7 (PR 058)	School of Pharmacy, University of Athens
38	Raw propolis Greece sample 8 (PR 059)	School of Pharmacy, University of Athens
39	Raw propolis Greece sample 9 (PR 060)	School of Pharmacy, University of Athens
40	Raw propolis Greece sample 10 (PR 061)	School of Pharmacy, University of Athens
41	Raw propolis Greece sample 11 (PR 062)	School of Pharmacy, University of Athens
42	Raw propolis Greece sample 12 (PR 063)	School of Pharmacy, University of Athens
43	Raw propolis Greece sample 13 (PR 064)	School of Pharmacy, University of Athens
44	Raw propolis Greece sample 14 (PR 065)	School of Pharmacy, University of Athens

8.3 List of ITS-sequences of investigated herbal substances from TCM (provided by the EDQM)

Acanthopanacis cortex (32961) (*Acanthopanax gracilistylus,* Araliaceae)

ACCGGGCGAGGGACGTGGGGTGCGCAAGTTCCCCAAGTCGCGAACCCATTGTCGGGGATCGCC
CTCGGGCGGTCCTCGACTGAACAACGTCACCCCGGCGCGGAATGCGCCAAGGAAATCAAACTG
AACTGAACGCGTCCCACCCGTTCGCGGGCTGTGGGGGCGTCTTTTTAAAACACAAACGACTCTC
GGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAAT
TGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGG
GCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCCCAACCCTGCACTCCCTCATGGGAGTC
ATGACTGAGGGGCGGATACTGGCCTCCCGTGTCTCACCGTGCGGTTGGCCCAAATGTGAGTCCT
TGGCGACGGACGTCACGACAAGTGGTGGTTGTAAAAAGCCCTCTTCTCCTGTCGTGCGGTGGCC
CGTCGCCAGCAAAAGCTCATGTGACCCTGTTGTGCCGTCCTCGACGAGCACTCCGACCGCGACC
CCAGGTCAGGCGGGACTACC (524 bp)

Acanthopanax giraldii harms cortex (32987), (*Acanthopanax gracilistylus*, Araliaceae)

GTTATCATACCGGGCGAGGGACGTGGGGTGCGCAAGTTCCCCAAGTCGCGAACCCATGGTCGG
GGATCGCCCTCGGGTGGTCCTCGACTGAACAACGTCACCCCGGCGCGGAATGCGCCAAGGAAA
TCAAACTGAACTGAACGCGTCCCACCCGTTCGCGGGCTGTGGAGGCGTCTTTTTAAAACACAAAC
GACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTG
GTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAG
GCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCCCAACCCTGCACTCCCTCAT
GGGAGTCATGACTGAGGGGCGGATACTGGCCTCCCGTGTCTCACCGCGCGGTTGGCCCAAATG
TGAGTCCTTGGCGACGGACGTCACGACAAGTGGTGGTTGTAAAAAGCCCTCTTCTCCTGTCGTG
CGGTGGCCCGTCGCCAGCAAAAGCTCATGTGACCCTGTTGTGCCGTCCTCGACGAGCACTCCGA
CCGCGACCCCAGGTCAGGCGGGACTACCCGCTG (607 bp)

Anemarrhenae rhizoma, 32483 (*Anemarrhena aspheloides*, Liliaceae)

CCATCCCTTTATATTAAGCTCCCTATTAAAGGCGANNCGTCTCTCGTGCCTCGGGCTTCGGCCCG
GCCGNNGGCCCCCGNGCTCCGTCCCGGGGCGGNGGGGCCGCNNCCGCCCCCGCGGCGCCTT
NGGCGTCGNGGNNCNCTGGTGCTGCCTTGCTCGGCGGNGCGGTCGGCCTGCCTTCCGCTCCC
CGCGCNGCGNTGTTNTCTTNNTNCNCNCGCCTCTCGGCNGCGGTTCTCTCGGCTCTCGCNTCGN
TGNNCGTCGTNNGCTGGTGCGCTTCCTGGTGTGTGTTGCTGCCTCCCGCGCCCCNTCGTGTTTT
TGNNCGCTTGTTGCGCCCGCGGCCTTCTGGCCGGGGGCTCGTCTGCCTGGGCGTCNCGCCNNN
NGCCCCTCCCCCCCCCCCCCCGGGGGGGGTCGTGGTGTCTGGCCCCCCGCGCCGCGNGGCGC
GGTGGGCCGNNGTTGGGGCTGCCGGCGGNTCGTGTCGGGCNCGGCTCGTGGTGGGCGCCTCC
TCGTTGTTCTCGGTGCN (520 bp)

Asari radix et rhizoma, 32540 (*Asarum heterotropoides* var. *mandshuricum*, *Asarum sieboldii* var. *seoulense*, *Asarum sieboldii*; Aristolochiaceae)

CCGAGCGTCGGTTGGACCAAGCGCTTGTTTGGTCCTCTCGACGCTTTGTCGACGCGCGTTCACT
CGAGTTCTTTTGGACCTTGTGAATGCGTCGTTGGCGCATTAACAACCCCCGGCACAATGTGTGCC

AAGGAAAACTAAACTCTAGAAGGCTCGTTTTCATGCTGCCCCCGTTCGCGGTGTGCTCATGGGAC
GCGGCTTCTTTATAATCACAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGA
ACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGC
AAGTTGCGCCCGAAGCCTTTTGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCC
CCCCACAATTCTCTGCAAAGGGAACCTGTGTTTTGGGGGCGGATATTGGTCTCCCGTGCTCATG
GCGTGGTTGGCCGAAATAGGAGTCCCTTCGACGGACGCACGAACTAGTGGTGGTCGTAAAAACC
CTCGTCTTTTGTTTCGTGCCGTCAGTCGCAAGGGAAACTNNNNNAAAACCCCAACGTGTTGTCTC
TTGACGACGCTTCGA (595 bp)

Coicis semen, 29251 (*Coix lacryma -jobi* var. *ma-yuen*, Gramineae)

CCGTCTCTCGTGCCTCGGGCTTCGGCCCGGCCGAAGGCCCCCGAGCTCCGTCCCGGGGCGGA
GGGGCCGCAACAGAACCCACGGCGCCTTAGGCGTCAAGGAACACTAATGCTGCCTTGCTCGGC
GGAGCGGTCGGCCTGCCTTCCGCTCCCCGCGCAGCGATGATATCTTAATACACACGACTCTCGG
CAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACCTGGTGTGAATTG
CAGAATCCCGCGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAGGCCTTCTGGCCAAGGGC
ACGTCTGCCTGGGCGTCACGCCAAAAGACACTCCCAACCCACCCCCGGGGAGGGACGTGGTGT
CTGGCCCCCCGCGCCGCAAGGCGCGGTGGGCCGAAGTTGGGGCTGCCGGCGAATCGTGTCGG
GCACAGCACGTGGTGGGCGACACCTAGTTGTTCTCGGTGCAGCGCCCCGGCACGCAGCCAGCA
CATCGGCCCTAAGGATCCATCGGGCACCGCAGCGCACCGTCGCTCGGACCGCGACCCCAGGTC
AGTCGGGACTACCCGCTGA (588 bp)

Coicis semen, 330102 (*Coix lacryma -jobi* var. *ma-yuen*, Gramineae)

TGAACCTGCGGAAGGATCATTGTCGTGACCCTTAAACAAAGCAGACCGCGAACCCGTCTCTCGT
GCCATCGGGCTTCGGCCCGGCCGAAGGCCCCCGAGCTCCGTCCCGGGGCGGAGGGGCCGCAA
CAGAACCCACGGCGCCTTAGGCGTCAAGGAACACTAATGCTGCCTTGCTCGGCGGAGCGGTCG
GCCTGCCTTCCGCTCCCCGCGCAGCGATGATATCTTAATACACACGACTCTCGGCAACGGATATC
TCGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACCTGGTGTGAATTGCAGAATCCCGC
GAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAGGCCTTCTGGCCAAGGGCACGTCTGCCTG
GGCGTCACGCCAAAAGACACTCCCAACCCACCCCCGGGGAGGGACGTGGTGTCTGGCCCCCCG
CGCCGCAAGGCGCGGTGGGCCGAAGTTGGGGCTGCCGGCGAATCGTGTCGGGCACAGCACGT
GGTGGGCGACACCTAGTTGTTCTCGGTGCAGCGCCCCGGCACGCAGCCAGCACATCGGCCCTA
AG (573 bp)

Daturae flos, 33002 (*Datura stramonium* var. *tatula*; Solanaceae)

GTGAACCTGCGGAAGGATCATTGTCGAAACCTGCAAAGCAGAACGACCCGCGAACCCGTTCAAA
CACTTGGGGAGCCGCGCGGGCGGGGCGCTTCGGCCCTCATCCGTGCGTCTCCCTCCCGTCCCC
GGCGCGCGCTCGCGGGCGCGCCGGGTGATGAACTAACCCCGGCGCGGAAAGCGCCAAGGAAT
ACTAAATTGATAGCCTGCCTCTCGCGCCCCGTTCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTT
TTGAAACAAAAACGACTCTCGGCAACGGATATCTGGGCTCTCGCATCGATGAAGAACGTAGCGAA
ATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCC
CAAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCCGCACTCC
GCGCCAAAAACCTTGGCTGCGGTTGTGTCGGGGGACGGATACTGGCCTCCCGTGAGCCCCCGA

GCCCGCGGCTGGCCTAAATGCGAGTCCACGTCGACGGACGTCACGGCAAGTGGTGGTTGGAAC
TCAACTCTCGTAATGTCGTGGCTACAGCCCGTCGCTCGTTTGTGCTCCTAGACCCTTCACGCGCT
TGGGCGCTCCGACCGC (653 bp)

Dioscoreae rhizoma, 33052 (*Dioscorea opposita*, Dioscoreaceae)

AAGGATCATTGTCGTGACCCTTAAACAAAGCAGACCGCGAACCCGTCTCTCGTGCCATCGGGCTT
CGGCCCGGCCGAAGGCCCCCGAGCTCCGTCCCGGGGCGGAGGGGCCGCAACAGAACCCACGG
CGCCTTAGGCGTCAAGGAACACTAATGCTGCCTTGCTCGGCGGAGCGGTCGGCCTGCCTTCCG
CTCCCCGCGCAGCGATGATATCTTAATACACACGACTCTCGGCAACGGATATCTCGGCTCTCGCA
TCGATGAAGAACGTAGCAAAATGCGATACCTGGTGTGAATTGCAGAATCCCGCGAACCATCGAGT
TTTTGAACGCAAGTTGCGCCCGAGGCCTTCTGGCCAAGGGCACGTCTGCCTGGGCGTCACGCCA
AAAGACACTCCCAACCCACCCCCGGGGAGGGACGTGGTGTCTGGCCCCCCGCGCCGCAAGGC
GCGGTGGGCCGAAGTTGGGGCTGCCGGCGAATCGTGTCGGGCACAGCACGTGGTGGGCGACA
CCTAGTTGTTCTCGGTGCAGCGCCCCGGCACGCAGCCAGCACATCGGCCCTAAGGATCCATCG
GGCACCGCAGCGCACCGTCGCTCGGACCGCGACCCCAGGTCAGTCGGGACTACCCGC (628 bp)

Ecliptae herba, 32971 (*Eclipta prostrata*, Asteraceae)

GAAGGATCATTGTCGAATCCTGCACGGCAGAACGACCTGTGAACATGTAAAACAATCGGCTTCAC
CGGGACTAAAGTTTTGCTTTGGTCCTCGTGAAGCATTGTTGACGTGTGTTCATGTTCGTCCCATTT
GGGCGTCATGGATATCAAGTTGACACAACAACCCCCGGCACGACATGTGCCAAGGAGAACTAAA
CTTAAAGAGCTTGTGTTATGACGCCCCGCTTTCGGTGTGCGCATTGCACATGGCTTCTTTGTAAA
CTCAAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGC
GATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAT
GCCATCTGGTTGAGGGCACGTCTGCCTGGGCGTCACGCATCACGTCGCCCCCACCACCATCCTC
ATTAGGGGAAGTGTTGTGTTGGGGCGGAGATTGGTCTCCTGCGCTAAGGGCGTGGTTGGCCTAA
ATAGGAGTCTTCTTACGAGAGACGCACGACTAGTGGTGGTTGATACGACAGTCGTCTCGTGTTGT
GCGTTTTAAATTGTGAGCGGATACTCTTGAAATACCCTAACGTGTTGTCTTGTGACGATGCC (644
bp)

Eupatorii herba, 32985 (*Eupatorium fortunei*, Compositae)

GTGAACGTGTATCAACAATATGGCTTGGCGGGTACTGACGCTTCTTGTTTCAATGCCCGTGAAGC
CCCGTCGACGTGCGCTCGTGGTGTGTCTTTCGGCACTCACGGCATCACGTTGACGCAACAACAA
CCCCCGGCACAGCACGTGCCAAGGAAAACCAAACTTAAGAGCGCCCGTGCCATGACGCCCCGT
GCTAGGTGTGTTCATGGTATGCGGCTTCTTTGTAATTCTAAACGACTCTCGGCAACGGATATCTC
GGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGA
ACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCACCCGGTTGAGGGCACGTCTGCCTGGG
CGTCACGCATCACGTCGCCCACACCAAACATCCATGATGTATGTGGGCGGAGACTGGTCTCCCG
TGCCCATGGCGCGGTTGGCCCAAATACGAGTCCGCTTAAGAGTGACGCACGACTGGTGGTGGTT
GATTACACAGTCGTCTCGTGTCGTGCGTCTCGATTCTTGACGGTATTAGCTCTTGTAGCACCCTA
ACGCGCCGTCTTGTGATGGCCCTTCGATCG (609 bp)

Eupatorii herba, 32499 (*Eupatorium fortunei*, Compositae)

GTGAACGTGTATCAACAATATGGCTTGGCGGGTACTGACGCTTCTTGTTTCAATGCCCGTGAAGC

CCCGTCGACGTGCGCTCGTGGTGTGTCTTTCGGCACTCACGGCATCACGTTGACGCAACAACAA
CCCCCGGCACAGCACGTGCCAAGGAAAACCAAACTTAAGAGCGCCCGTGCCATGACGCCCCGT
GCTAGGTGTGTTCATGGTATGCGGCTTCTTTGTAATTCTAAACGACTCTCGGCAACGGATATCTC
GGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGA
ACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCACCCGGTTGAGGGCACGTCTGCCTGGG
CGTCACGCATCACGTCGCCCACACCAAACATCCATGATGTATGTGGGCGGAGACTGGTCTCCCG
TGCCCATGGCGCGGTTGGCCCAAATACGAGTCCGCTTAAGAGTGACGCACGACTGGTGGTGGTT
GATTACACAGTCGTCTCGTGTCGTGCGTCTCGATTCTTGACGGTATTAGCTCTTGTAGCACCCTA
ACGCGCCGTCTTGTGATGGCCCTTCGATCGC (610 bp)

Evodiae fructus, 32508 (*Evodia rutaecarpa*, *Evodia rutaecarpa* var. *officinalis*, *Evodia rutaecarpa* var. *bodinieri*; Rutaceae)

ACACCGGTGGGGGCGTGCTTCGCGGCCGCTCCCCCTGCCCCCGTGGGTGCGGGACTCGTCCT
GTTCCCCCCGGGGGCGACCAACTAACCCCCGGCGCGGACTGCGCCAAGGAAATCTAACGAGAG
AGCACGCTCCCAGGGCACCGGACATGGTGATCCCCAGGATGCGGCGCCTTCTTTCACTTTATCT
ATAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATA
CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCCAAGCCT
TTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCATCGTTGCCCCACCCCCACCCCCACCCCG
GGGGCCTGGCGGTGCGGGCGGATAATGGTCTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAA
TTCGAGTCCTTGGCGACCGGAGCCGCGACAATCGGTGGTGAAAAGCCTCTCGAGCTCTAGTCGC
GAGCCCGTGTCTCTGTTTCAGGACTCAGGGACCCTGATGCTCCGCGCAAGCGGTGCTCGCATCG
CGACCCCAGGTCAGGCGGGACTACC (598 bp)

Farfare flos, 32518 (*Tussilago farfara*, Compositae)

CGAAACCTGCATAGCAAAATGACCTGTGAACATGTTACAACAATCTTGTCTCCTTAGAGTTGGGCT
CTTGTTCGATTCTTTGGATTCCTTGTCGATGTGCATCTTTGGTTAGCCTATCCGGTTCTAAAGATG
TTATGTTGGCCTAATAACAACCCCCGGCACGGCATGTGCCAAGGAAAATCAAACTTAAAAAGGGC
TCGTATCAAGCTCCCCCGTTTGCGGGGTTTTGCATGGGACGTGGCTTCTTTGTAATAATAAACGA
CTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTG
TGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCAAAGCCTTTTGGCT
GAGGGCACGTCTGCCTGGGCGTCACATATCGCGTCGCCCCTACCATGCCTTCTTGATAGGGATG
CTTGGATTGGGGCGGAGATTGGTCTCCCGTTCCTATGGTGCGGTTGGCTAAAACTGGAGTCCTC
TTCAACGGACGCATGATTAGTGGTGGTTGAGAAGACCCTCTTATCAAGTTGTGTGTTCCAAGGAG
TAGGGAATATCTCTTTAATGACCCTTGTCGTTTTTAGACGATGATTTGACCGCGACCCCAGGTCAG
GCGGGACTACCCGCT (666 bp)

Farfare flos, 32007 (*Tussilago farfara*, Compositae)

GATCATTGTCGAAACCTGCATAGCAAAATGACCTGTGAACATGTTACAACAATCTTGTCTCCTTAG
AGTTGGGCTCTTGTTCGATTCTTTGGATTCCTTGTCGATGTGCATCTTTGGTTAGCCTATCCGGTT
CTAAAGATGTTATGTTGGCCTAATAACAACCCCCGGCACGGCATGTGCCAAGGAAAATCAAACTT
AAAAAGGGCTCGTATCAAGCTCCCCCGTTTGCGGGGTTTTGCATGGGACGTGGCTTCTTTGTAAT
AATAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGA

TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCAAAGC
CTTTTGGCTGAGGGCACGTCTGCCTGGGCGTCACATATCGCGTCGCCCCTACCATGCCTTCTTG
ATAGGGATGCTTGGATTGGGGCGGAGATTGGTCTCCCGTTCCTATGGTGCGGTTGGCTAAAACT
GGAGTCCTCTTCAACGGACGCATGATTAGTGGTGGTTGAGAAGACCCTCTTATCAAGTTGTGTGT
TCCAAGGAGTAGGGAATATCTCTTTAATGACCCTTGTCGTTTTTAGACGATGATTTGACCGCGACC
CCAGGTCAGGCGGGACTACCCG (673 bp)

Farfare flos, 33043 (*Tussilago farfara*, Compositae)

GGATCATTGTCGAAACCTGCATAGCAAAATGACCTGTGAACATGTTACAACAATCTTGTCTCCTTA
GAGTTGGGCTCTTGTTCGATTCTTTGGATTCCTTGTCGATGTGCATCTTTGGTTAGCCTATCCGGT
TCTAAAGATGTTATGTTGGCCTAATAACAACCCCCGGCACGGCATGTGCCAAGGAAAATCAAACT
TAAAAAGGGCTCGTATCAAGCTCCCCCGTTTGCGGGGTTTTGCATGGGACGTGGCTTCTTTGTAA
TAATAAACGACTCTCGGCAACGGATATCTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCG
ATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCAAAG
CCTTTTGGCTGAGGGCACGTCTGCCTGGGCGTCACATATCGCGTCGCCCCTACCATGCCTTCTT
GATAGGGATGCTTGGATTGGGGCGGAGATTGGTCTCCCGTTCCTATGGTGCGGTTGGCTAAAAC
TGGAGTCCTCTTCAACGGACGCATGATTAGTGGTGGTTGAGAAGACCCTCTTATCAAGTTGTGTG
TTCCAAGGAGTAGGGAATATCTCTTTAATGACCCTTGTCGTTTTTAGACGATGATTTGACCGCGAC
CCCAGGTCAGGCGGGACTACCCGCTGAGTTTTAAGCAATG (691 bp)

Gardeniae fructus, 33031 (*Gardenia jasminoides*, Rubiaceae)

GTGCCGAACCCCGGGCGCCGGGGGAGGGGGCGGCGCCCTCGCCCTCCCTTTCCCCGTCGCCC
TCCCGTGCGCCCGCCGCGCGGACCGACGAACCAACCCCGGCGCGGAAAGCGCCAAGGAAAAC
TCAAAACGGATCGCCCGGCCCCCGACCGCCCCGTCCGCGGAGCGCGGGAGGGGGCGCCGCG
GCGCCTGTCGTAACCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAAC
GTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCAAGTCTTTGAACGCAA
GTTGCGCCCGAAGCCATCAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCAC
CCCCCTCCCGCGGGGGCGGCGGAGACTGGCCTCCCGTGCCCCGGGGCGCGGCCGGCCCAAAT
GAGAGTTCCTCGGCGAGGGGCGTCACGACTGGTGGTGGTTGAGTCCCTCAACTCGAGTCGTCG
TCGTGCCGGCAAACCCCAGCCGCGGTCCCGTGACCCCGAAGCTCCCGCGAGCCTCGACCGCGA
CCCCAGGTCAGGCGGGACTACCCGCTGNGTTTAAGC (601 bp)

Gardeniae fructus praeparata, 33024 (*Gardenia jasminoides*, Rubiaceae)

CAGACGACCGCGAACCAGTGCCGAACCCCGGGCGCCGGGGGAGGGGGCGGCGCCCTCGCCCT
CCCTTTCCCCGTCGCCCTCCCGTGCGCCCGCCGCGCGGAACGATGAACCAACCCCGGCGCGGA
AAGCGCCAAGGAAAACTCAAAACGGATCGCCCGGCCCCCGACCGCCCCGTCCGCGGAGCGCG
GGAGGGGGTGCCGCGGCGCCTGTCGTAACCAAAACGACTCTCGGCAACGGATATCTCGGCTCT
CGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATC
AAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATCAGGCCGAGGGCACGTCTGCCTGGGCGTCA
CGCATCGCGTCGCCACCCCCCTCCCGCGGGGGCGGCGGAGACTGGCCTCCCGTGCCCCGGGG
CGCGGCCGGCCCAAATGAGAGTTCCTCGGCGAGGGGCGTCACGACTGGTGGTGGTTGAGTCCC

TCAACTCGAGTCGTCGTCGTGCCGGCAAACCCCAGCCGCGGTCCCGTGACCCCGAAGCTCCCG
CGAGCCTCGACCGCGACCCCAGGTCAGGCGGGACTACCCGCTG (609 bp)

Houttuyniae herba, 32984 (*Houttuynia cordata*, Saururaceae)

TTCGTTCATCTATCACGTAAGCGCCCAAGCAAGCGGACGGCGGCGAGGATCGTTCTTCGTGCGG
GGCGTCTCGCCCCCGCGACCAGCCCCTCCCGTTGGCCGCCCTTGTGTGTGCCAAACACAAACC
CCGGCGCTTCGCGCGCCAAGGAAATCAAATCTGTCAAGTGCGACTCCCGGGGGCCCGGAGACG
GTGTCCCCCGGGCGTCGTCGCTGCACATTACCATATTACAATGACTCTCGGCAACGGATATCTCG
GCTCTTGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
CCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGCCTGCCTGGGT
GTCACACATCGTTACCCAAACGCAAATTATTGCCTCGGGTGCATGCTGACCTCCCGCGAGCACC
GTTTCGTGGTTGGTTGAAAATCGAGTCCGTGGCCGAGTTCGCCGTGACAAAATGGTGGATGAGT
TCGTCACAGACGACGCTCGAGACCAGTCACGCGTGACTCCGTCGTTCCCGGACTCCCCGACCCT
GTGCGTGCGTCTCTACCCGACGACGCAGGCTATCAACGAGACCTCAGGTCAGGCGGGACTACC
CGC (642 bp)

Isatidis radix, 258471 (*Isatis indigotica*, Cruciferae)

TCGTAAACAGAACGACCCGCGAACGATTGATCATCACTCTCGGTGGGCTGGTGTCTTAGCTGATT
CCGTGCCTGCCGATTCCGTGGTTATGCGCGTGGTCTCAGCCAAGATTCATATCTCGGTTGGGTC
ATGCGCCTAGCTTCCGGATATCACCAAACCCCGGCACGAAAAGTGTCAAGGAACATGCAACTAAA
CAGCCTGCGTTCGCCTACCCGGAGACGGTGTTTGCGTGGACGCTGTGCTGCAATCTAAAGTCTA
AAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATA
CTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCTAAGCCT
TCTGGCCGAGGGCACGTCTGCCTGGGTGTCACAAATCGTCGTCCCCCCATCCTCTCGAGGATAA
TGGACGGAAGCTGGTCTCCCGTGTGTTACCGCACGCGGTTGGCCAAAATCCGAGCTAAGGACGC
AAGGAGCGTCTCGACATGCGGTGGTGAATTAAAACCTCGTCATACCGTTGGCCGCTCCTGTCCT
GATGCTCTCGATGACCCAAAGTCCTCAACGC (610 bp)

Lycii fructus, 32494 (Lycium barbarum, Solanaceae)

GGAGCCGCGCGGGCGGGGTGCTTCGGCCCCCCGTGTGCGCGTCTCCCCCCTCGTCCCCGGCG
CGCGCGCCCGCGCGCGCGTCGGGTGACTAACGAACCCCGGCGCGAAAAGCGCCAAGGAATACT
TAAATTGATAGCCTGCCTCTCGCGCCCCGTCCGCGGTGCGCGCGGGAGGGCCTGTGCTTCTCTT
GAAACAGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAA
TGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC
GAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCCCGCGCACC
GCGCCCATGCTCTGGGTCGCGGTGGTGTCGCGGGGCGGATACTGGCCTCCCGTGCGCCTCGC
GCTCGCGGCCGGCCTAAATGCGAGTCCACGTCGACGGACGTCACGGCAAGTGGTGGTTGTAAC
CCAACTCTCGAAGTGTCGTGGCCATACCCCGTCGCGCGTTTGGCCTCCCGGACCCTTCTTGCGC
TTNGGCGCTCCGACCGC (587 bp)

Lycii fructus, 32989 (Lycium barbarum, Solanaceae)

TTTCCGTANNGTGAACCTGCGGAAGGATCATTGTCGAAACCTGCACAGCAGAACGACCCGCGAA
CGCGTTTCAACACTGGGGAGCCGCGCGGGCGGGGTGCTTCGGCCCCCCGTGTGCGCGTCTCC

CCCCTCGTCCCCGGCGCGCGCGCCCGCGCGCGCGTCGGGTGACTAACGAACCCCGGCGCGAA
AAGCGCCAAGGAATACTTAAATTGATAGCCTGCCTCTCGCGCCCCGTCCGCGGTGCGCGCGGGA
GGGCCTGTGCTTCTCTTGAAACAGAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGAT
GAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTG
AACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCG
TCGCCCCCCGCGCACCGCGCCCATGCTCTGGGTCGCGGTGGTGTCGCGGGGCGGATACTGGC
CTCCCGTGCGCCTCGCGCTCGCGGCCGGCCTAAATGCGAGTCCACGTCGACGNANGTCACGGC
AAGTGGTGGTTGTAACCCAACTCTCGAAGTGTCGTGGCCATACCCCGTCGCGCGTTTGGCCTCC
CGGACCCTTCTTGCGCTTAGGCGCTCCGACCGC (667 bp)

Salviae miltiorrhizae radix et rhizoma, 32482 (*Salvia miltorrhiza*, Lamiaceae)

GTGTTTACAATGCCGGGTGCGTGGCGTGGGGGCAACCCCCGTCATGTACTCGGTCCCCCGCCG
GCGCGCGTCCTCGGGCAGTGTCGTGCGGGCTAACGAACCCCGGCGCGGAATGCGCCAAGGAA
AACTAATCGAAGCGTCCGCCCCTCGTGCCCCGTTCGCGGTGCGCGCGGGGGGATTGGATGTCT
ATCAAATGTCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCG
AAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCG
CCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCCCTCCC
CGCGCATAGCGTGGGCTGCGGGGGCGGAAACTGGCCTCCCGTGCGCCCCGGCGCGCGGCTGG
CCCAAATGCGATCCCTCGGCGACTCGTGTCGCGACAAGTGGTGGTTGAACAACTCACTTTCATGT
CGTGATTCTGCGTCGTCGGTATGGGCATCTGTAAACGACCCAACGGTGTAGGCGCCACACGGCG
CCCAACCTTCGACCGCGACC (592 bp)

Salviae miltiorrhizae radix et rhizoma, 33040 (*Salvia miltorrhiza*, Lamiaceae)

AACCTGCGGAAGGATCATTGTCGAAACCTGCAAAGCAGACCGTGAACACGTGTTTAACAATGCCG
GGTGCGTGGCGTGGGGGCAACCCCCGTCATGTACTCGGTCCCCCGCCGGCGCGCGTCCTCGG
GCAGTGTCGTGCGGGCTAACGAACCCCGGCGCGGAATGCGCCAAGGAAAACTAATCGAAGCGT
CCGCCCCTCGTGCCCCGTTCGCGGTGCGCGCGGGGGGATTGGATGTCTATCAAATGTCAAAAC
GACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTG
GTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAG
GCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCCCTCCCCGCGCATAGCGTGG
GCTGCGGGGGCGGAAACTGGCCTCCCGTGCGCCCCGGCGCGCGGCTGGCCCAAATGCGATCC
CTCGGCGACTCGTGTCGCGACAAGTGGTGGTTGAACAACTCACTTTCATGTCGTGATTCTGCGTC
GTCGGTATGGGCATCCGTAAACGACCCAACGGTGTAGGCGCCACACGGCGCCCAACCTTCGAC
CGCGA (640 bp)

Schisandrae chinensis fructus, 32514 (*Schisandra chinensis*, Magnoliaceae)

TGACCGGCGAACTTGTGATATTGTTCCTTGAGGGGTGACGGTCTATCCTTGTGATGCCGAAAACC
CTTCCCCTCTCATTGCTACCTTGTATGACATGCTTTGGTCTTGTGCCATGCGTTGATCCTTGGGTG
CAACACGAGGGAATGAACCAACAACACCGGCGCGGCATGCGTCAAGTAATAACAAACTGAATAG
GCACTCCCCCCTGTTGTGTCCCTCAAGGGTGTGGCTTGGGGGTGTTGCCACTTATTCTAATTGAA
AACATAAATGACTCTCGGCAACGGATATCTAGGCTCTTGCCACGATGAAGAACGTAGCGAAATGC
GATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAG

GCCACCTGGCCAAGGGCACGCCTGCCTGGGCGTCACGCTTTGCGTCGCTCCCCTCCCTCCCATT
CTCCTTTTTTGGTGTATGGTGTTTGTGAGGAGCGGATATTGGCTGCCCGTGCCATGTTTGTGCGG
TCGGCCGAAAGATGGGCCCCTGGTGTGTTGTGACACGACGNGTGGTGGTCAAATGCCCTTCTCA
CCGCGTGGGACGTNNNGTCGCATTCCTTGTGGCTCTTGGGACTCTTGGAGCCGCTTCACGGCA
(646 bp)

Spica prunellae, 32524 (*Prunella vulgaris*, Lamiaceae)

ACCTGCGGAAGGATCATTGTCGAAACCTGCAAAAGCAGACCGCGAACACGTGCTTAACTACACG
GCGCGCGGCGCGGGGGACGCGAGTCTCCCCGTCGTGCGCTGAATCCCCGCCGGCGCGCTCCC
TCGGGTCGCGTCGTTCGGGCTAACGAACCCCGGCGCGGAATGCGCCAAGGAAAACTTAACGAA
GCGTCCGCATCCCCGCAGCCCGTCCGCGGAACCTGCGGGGGGACCGGTCGTCTATCATAATGT
CAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA
TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGC
CTTTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCACCTCACCGCGCAC
GAACGTGCCGGCGAGTGGGGCGGATATTGGCCCCCCGTGCGCCTCGGCGTGCGGTCGGCCCA
AATGCGATCCCTCGGCGACTCGTGTCGCGACTAGTGGTGGTTGAACCTCAATCTCTCAATCGTCG
TGCTCCCGCGTCGTCTGCAAGGGCATCAATGAACGACCCAACGGTGTCGGTGCCGCACGGCGC
CCCACCTTCGACCGC (649 bp)

Spica prunellae, 32986 (*Prunella vulgaris*, Lamiaceae)

ACCTGCGGAAGGATCATTGTCGAAACCTGCAAAAGCAGACCGCGAACACGTGCTTAACTACACG
GCGCGCGGCGCGGGGGACGCGAGTCTCCCCGTCGTGCGCTGAATCCCCGCCGGCGCGCTCCC
TCGGGTCGCGTCGTTCGGGCTAACGAACCCCGGCGCGGAAAGCGCCAAGGAAAACTTAACGAA
GCGTCCGCATCCCCGCATCCCGTCCGCGGAACCTGCGGGGGGACCGGTCGTCTATAGTAATGT
CAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGA
TACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGC
CTTTAGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCACCTCACCGTGCAC
GAACGTGCCGGCGAGTGGGGCGGATATTGGCCCCCCGTGCGCCTCGGCGTGCGGTCGGCCCA
AATGCGATCCCTCGGCGACTCGTGTCGCGACTAGTGGTGGTTGAACCTCAATCTCTNAATCGTCG
TGCTCCCGTGTCGTCTGCAAGGGCATCAATGAACGACCCAACGGTGTCGGTGCCGCACGGCGC
CCCACCTTCGAC (646 bp)

Zanthoxyli pericarpium, 32526 (*Zanthoxylum schinifolium*, Rutaceae)

GGGGCATGTTCAACNTACTGCGAGAGCAGAACGACCCGCGACCCCG~GTCAACA~TCGTGGGG
GGGCGGCGCGCTTCGCTCGCCGCACCCCCACGTCTCCGCGGGTGCGGGACCAGTCCCGTTCC
CCGCGGGGGCGAACAACGAACCCCCGGCGCGGTCTGCGCCAAGGAAATCTAACGAGAGAGCAC
GCTCTCAGGGCCCCGGACACGGTGTGCTCTGGGACGCGGCGCCTTCTTTCACTCTATCTATAAC
GACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTG
GTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAG
GCCGAGGGCACGTCTGCCTGGGTGTCACGCATCGTTGCCCCACCCCACCCCCTCAGGGGGGCC
CGGCGGTGCGGGCGGATAATGGCCTCCCGTGCGCTCCCCGCTCGCGGCTGGCCCAAAATTTGA
GTCCCTGGCGACCGGAGCCGCGACGATCGGTGGTGAAAACAAACC (552 bp)

Zanthoxyli pericarpium, 32952 (*Zanthoxylum schinifolium*, Rutaceae)

GGGGCGATATCGACTCTGCAGAGCAGAACGACCCGCGACCCCAGGTCAACCTANCGGGGGGCG
CGCTTCGCGGCCGCTCCCCCACGTCTCCGCGGGTGTGGGACTCCTCCCGTTCCCCGCGGGGGC
GGATAACGAACCCCCGGCGCGGAATGCGCCAAGGAAATCTAACGAGAGAGCACGCTCCCGGGG
CCCCGGACACGGTGTGCTCCGGGACGCGTCGCCTTCTTTCACTCTATCTGAAACGACTCTCGGC
AACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGC
AGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCCAAGCCTTTAGGCCGAGGGCA
CGTCTGCCTGGGTGTCACGCATCGTTGCCCCGCCCCACCCCCGCCCGGGGGCTTGGCGGCGA
GGGCGGATAATGGCCTCCCGTGCGCTCCCCGCTCGCGGTTGGCCCAAATTCGAGTCCCCGGCG
ACCGGAGCCGCGACGATCGGTGGTGAAAACAAACCTCTCGAAC (550 bp)

Ziziphi spinosae semen, 32509 (*Ziziphus spinosa*, Rhamnaceae)

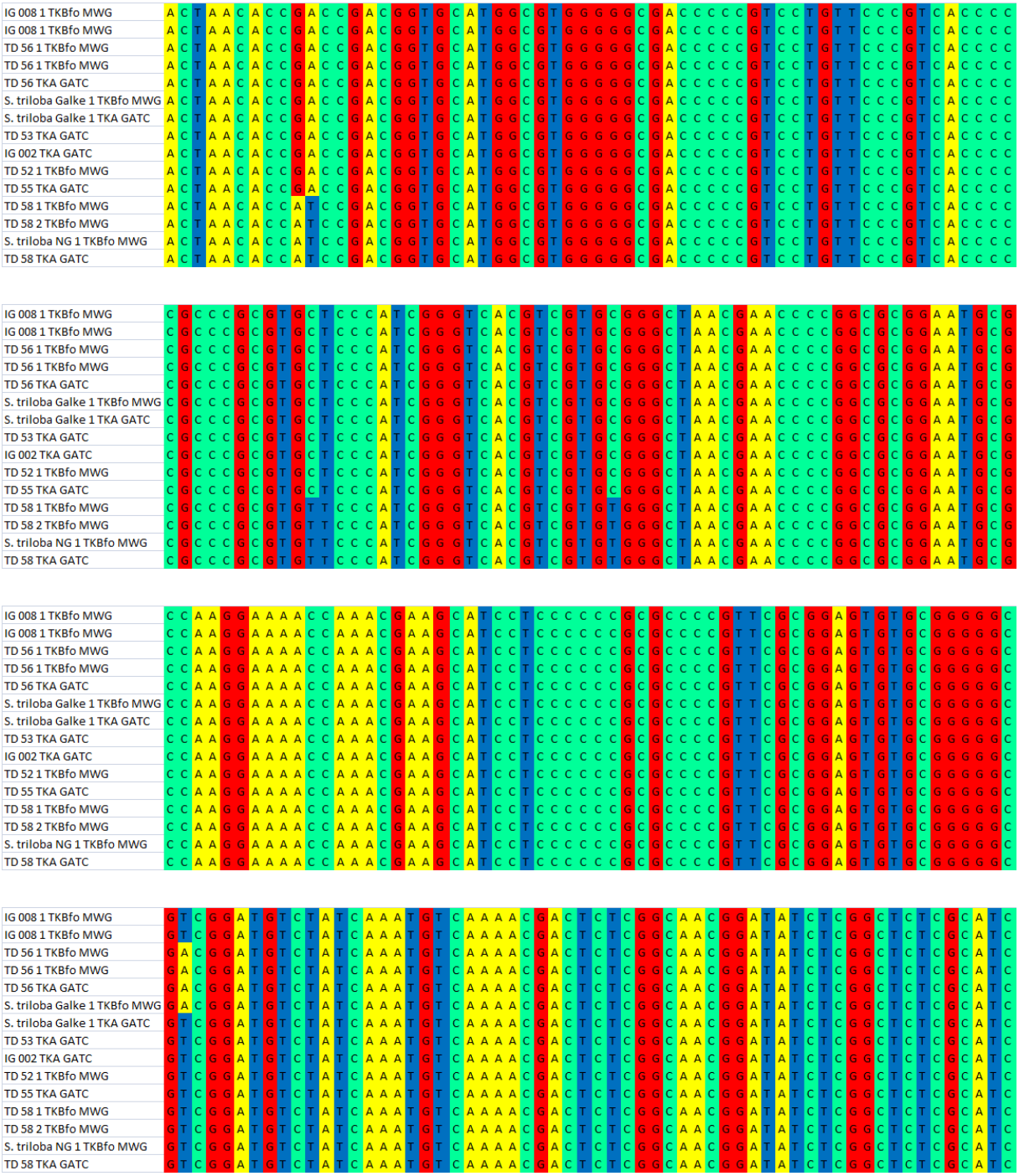
ACCTGCGGAAGGATCATTGTCGAAACCTGCCCAGCAGAACAACCAGCGAACCCGTGAATAACAC
ATCGGGGACCCCGGGGCCCTGTGTCCCGGAGCCTTCCTTGGTCGGGGGTCTGCACCTCGCGCC
TCGCCCGTCGATGCGCGGTTGCAGCCTTCCCGGCCGCACAAACGAACCCCGGCGCAAACCGCG
CCAAGGAACACCTAACGAATTGGCATTCACCCCCCGCCCCGGAGACGGTGTGCGGTCGGGGTG
TGCGTCGTATTCTCTATTGTAATGTCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATC
GATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTT
TTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACACAAC
GTTGCCCCCCATCCCAACCTCGACCTCGAGGCGAAGAGGGGGCGGATGCTGGCCTCCCGTGTG
CCACGGTCCGCGGCTGGCTGAAATACGGGTCCCCGGCGACGAGTGCCGCAGCAATCGGTGGTT
GTCCAACCCTCGGCTCCCTGCTGCGTGCGCGGATCGCTGTCGCGGCCCTACAGAGACCCCAAT
GCGCTGCCAATGCGGCGTCTCCAACG (661 bp)

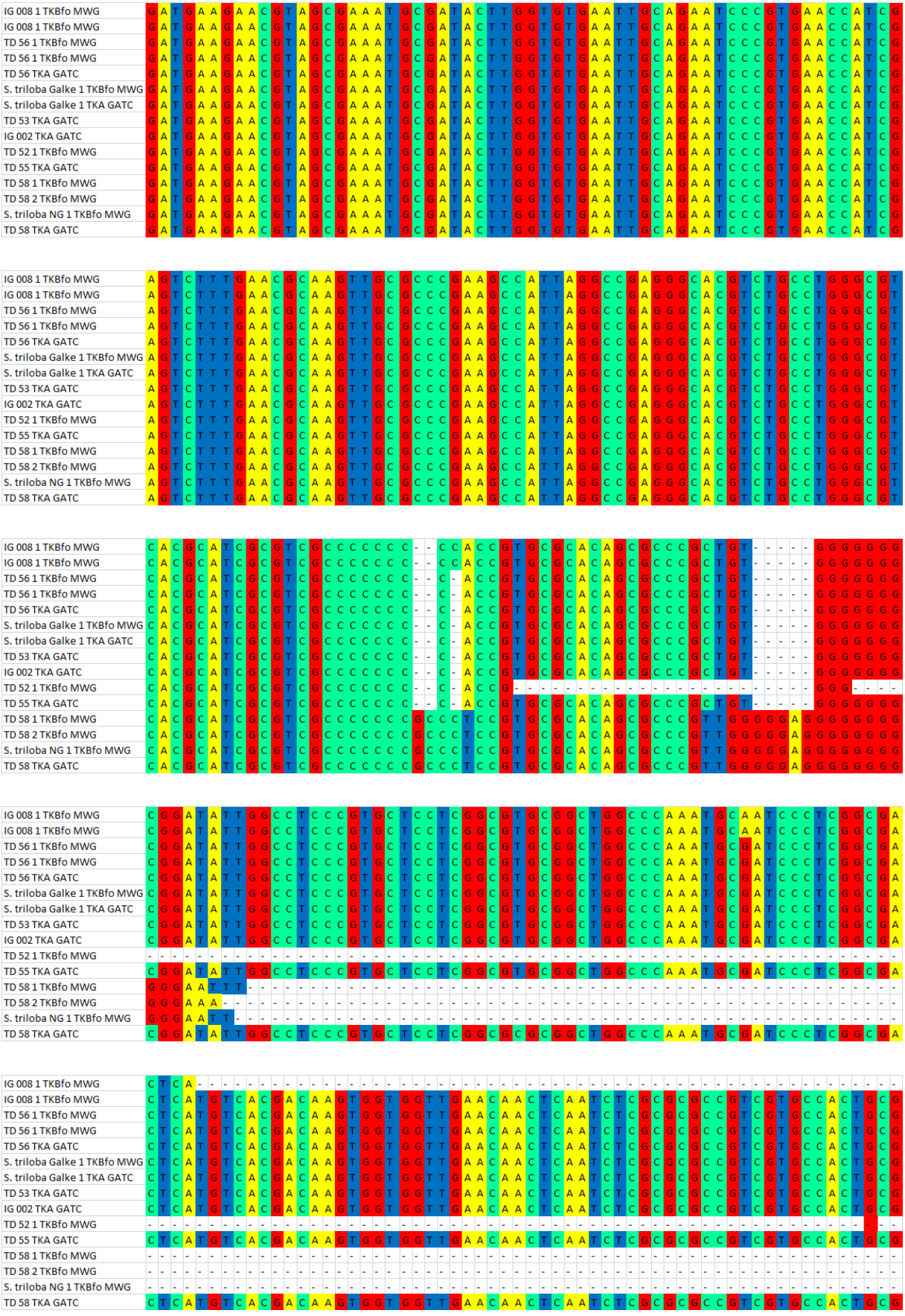
Ziziphi spinosae semen, 33011 (*Ziziphus spinosa*, Rhamnaceae)

ACCTGCGGAAGGATCATTGTCGAAACCTGCCCAGCAGAACAACCAGCGAACCCGTGAATAACAC
ATCGGGGACCCCGGGGCCCTGTGTCCCGGAGCCTTCCTTGGTCGGGGGTCTGCACCTCGCGCC
TCGCCCGTCGATGCGCGGTTGCAGCCTTCCCGGCCGCACAAACGAACCCCGGCGCAAACCGCG
CCAAGGAACACCTAACGAATTGGCATTCACCCCCCGCCCCGGAGACGGTGTGCGGTCGGGGTG
TGCGTCGTATTCTCTATTGTAATGTCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATC
GATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTT
TTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGCGTCACACAAC
GTTGCCCCCCATCCCAACCTCGACCTCGAGGCGAAGAGGGGGCGGATGCTGGCCTCCCGTGTG
CCACGGTCCGCGGCTGGCCGAAATACGGGTCCCCGGCGACGAGTGCCGCAGCAATCGGTGGTT
GTCCAACCCTCGGCTCCCTGCTGCGTGCGCGGATCGCTGTCGCGGCCCTACAGAGACCCCAAT
GCGCTGCCAATGCGGCGTCTCCAAC (660 bp)

8.4 *Complete alignments of the ITS region of different samples of S. officinalis and S. triloba*

(section of the alignments is shown in 4.1.2.3 and figure 20)

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IG 008 1 TKBfo MWG
TD 56 1 TKBfo MWG
TD 56 1 TKBfo MWG
TD 56 TKA GATC
S. triloba Galke 1 TKBfo MWG
S. triloba Galke 1 TKA GATC
TD 53 TKA GATC
IG 002 TKA GATC
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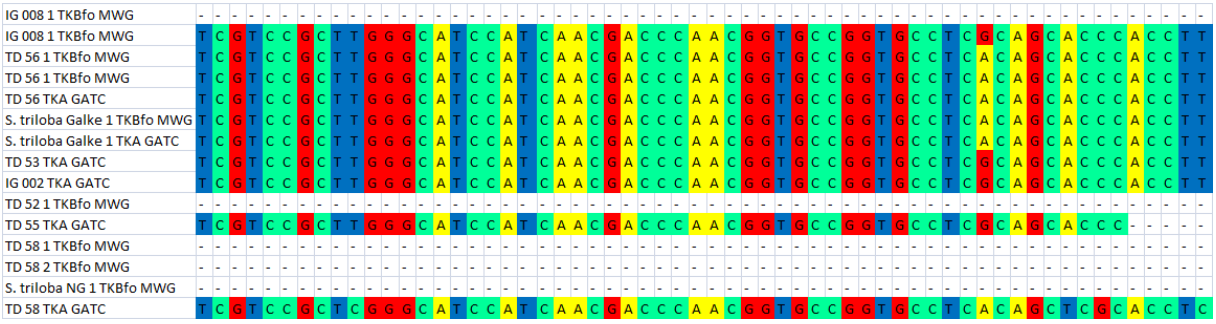
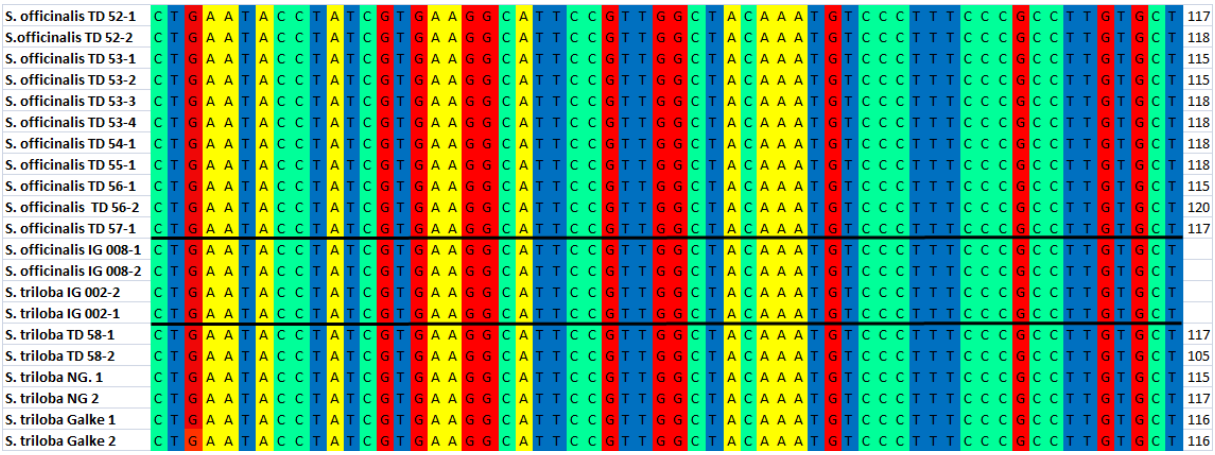
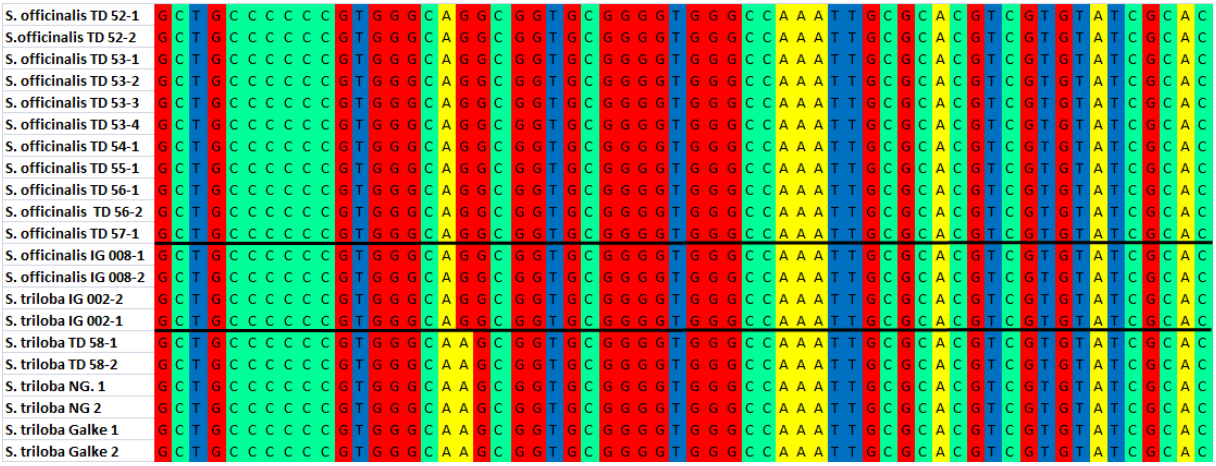
S. triloba NG 1 TKBfo MWG
TD 58 TKA GATC

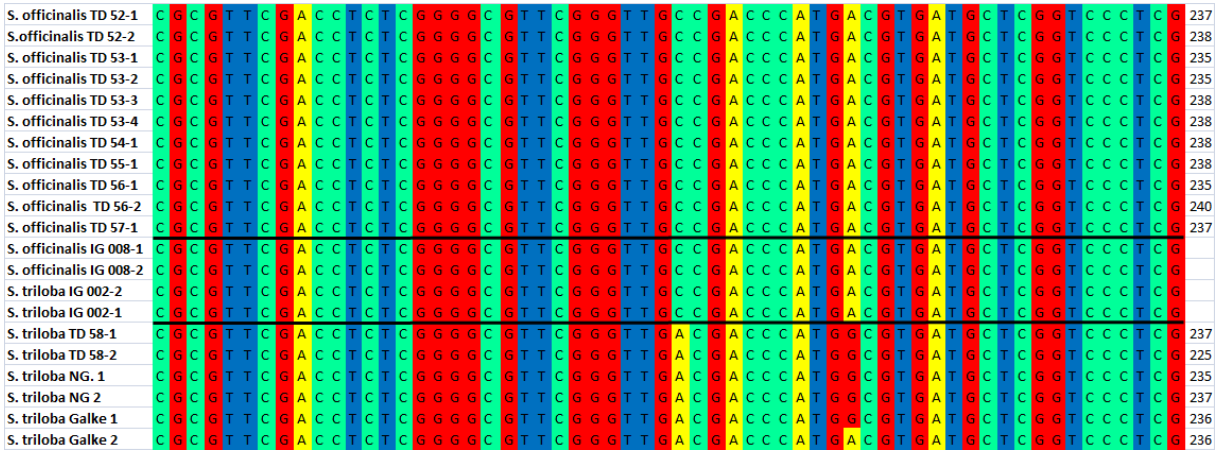


8.5 *Complete alignments of the ETS region of different samples of S. officinalis and S. triloba*

(section of the alignments is shown in 4.1.2.3 and figure 21).



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S. officinalis TD 53-2 C G C G T T C G A C C T C T C G G G G C G T T C G G G T T G C C G A C C C A T G A C G T G A T G C T C G G T C C C T C G 235
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S. officinalis TD 54-1 C G C G T T C G A C C T C T C G G G G C G T T C G G G T T G C C G A C C C A T G A C G T G A T G C T C G G T C C C T C G 238
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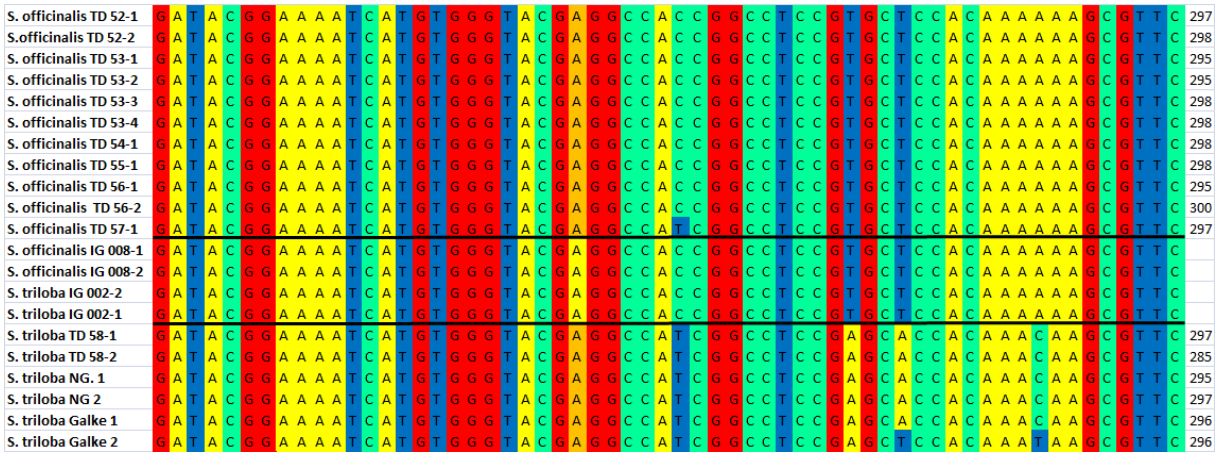
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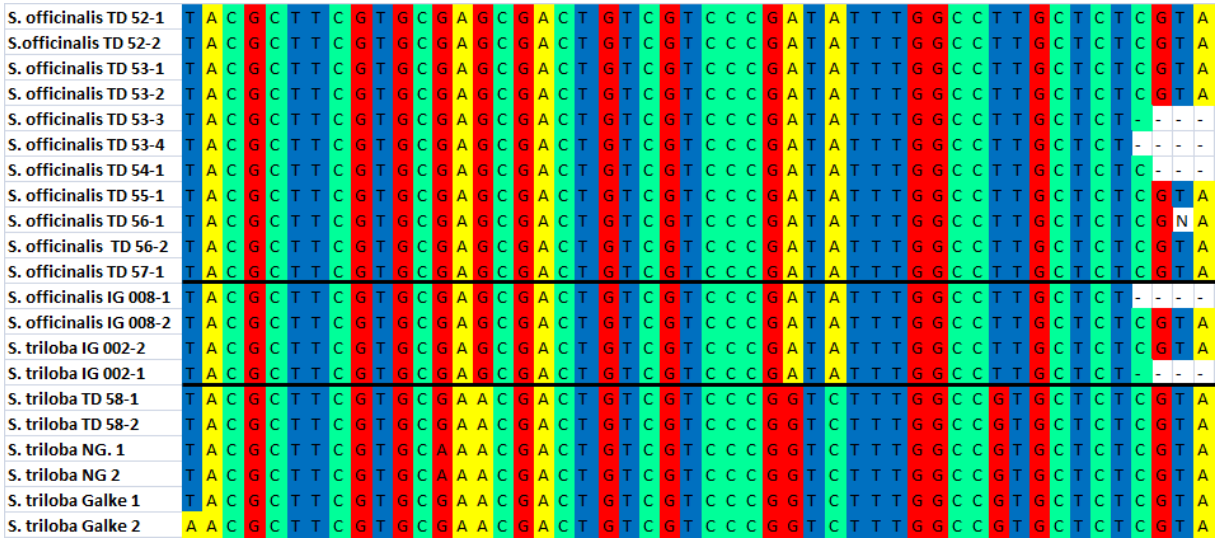
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