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Phenolic compounds as marker compounds for botanical origin determination of German propolis samples based on TLC and TLC-MS

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Summary

Propolis, a complex mixture of different plant exudates collected and processed by honeybees, contains a wide range of bioactive substances. German propolis has so far only rarely been studied with respect to its constituents and botanical origin. To investigate the composition of specific phenolic constituents in bud extracts and German propolis, TLC and TLC-MS methods were developed. Ethanolic extracts from representative propolis samples and tree buds were analyzed by TLC. Compound assignment was achieved through mass spectrometric detection, and TLC using the DPPH assay was used to assess the antioxidant capacity.

According to their TLC fingerprints, propolis samples were classified into three types (O/B/G) based on the results of former investigations. Identification of flavonoid aglycones and their methyl esters as well as acetates or butyrates was possible in both bud exudates of black poplar hybrids (*Populus* × *canadensis* Moench 'Robusta') and O-type propolis with TLC-MS. In aspen (*Populus tremula* L.) and B-type propolis, phenolic acid glycerols were detected as common constituents. Based on the marker compounds identified in the present study, black poplar hybrids and aspens were identified as major sources of the investigated propolis samples whereas horse chestnut (*Aesculus hippocastanum* L.) and birch (*Betula pendula* Roth) were of minor importance. The O-type propolis displayed superior antioxidant activity, but also contained higher amounts of potential allergens like caffeic acid derivatives than B-type propolis.

Introduction

Propolis, often referred to as bee glue, is a sticky material produced by honeybees from various plant resins and bud exudates. Bees use propolis mainly to protect their hive from adverse weather conditions or invaders by sealing internal walls, holes, and cracks of the beehive or to embalm dead insects in order to prevent hive infections.

Mankind has been using propolis for ages, and especially in traditional medicine the application of propolis has a long history with first reports dating back to 300 BC (GHISALBERTI, 1979). Today it is still used in folk medicine, but has also become a popular ingredient in so-called health food and drinks or in natural cosmetics. This popularity is due to its putative health-beneficial effects, based on data from numerous research activities in the past decades identifying anticancer, antioxidant, anti-inflammatory, antibiotic, antifungal, and antihepatotoxic effects of propolis (BURDOCK, 1998; KUJUMGIEV et al., 1999; BANSKOTA et al., 2000, 2001a, b, 2002; KARTAL et al., 2003; CHEN et al., 2004; BOYANOVA et al., 2005; GREGORIS et al., 2010; BARBARIĆ et al., 2011; SOBOČANEC et al., 2011). However, a sensitizing or even allergenic potential has also been reported (HAUSEN et al., 1992; GIUSTI et al., 2004), which is partly caused by certain caffeic acid derivatives like caffeic acid benzyl ester or caffeic acid phenethyl ester, i.e. compounds which, at the same time, are associated with the aforementioned health-beneficial effects (BANSKOTA et al., 2001b).

The composition of propolis especially with regard to its botanical origin has been the subject of numerous scientific studies, revealing a complex, heterogenous compound profile: Propolis is composed of waxes, resins, balsams, essential oils, pollen, and other organic substances like labdane diterpenes, cycloartane triterpenoids, phenolic acids and other phenolic compounds (GHISALBERTI, 1979; BANKOVA et al., 2006; MOREIRA et al., 2008). Since the biosynthesis of plant secondary metabolites like flavonoids and phenolic acid derivatives is genetically determined, these compounds may serve as chemotaxonomic markers (WOLLENWEBER, 1989).

The botanical sources of propolis strongly depend on climate and specific vegetation characteristics. Poplar species have been reported to be the main source for propolis from temperate zones, e. g. European propolis from Albania, Bulgaria, Greece, Hungary, Italy, Russia, Malta, the Netherlands, Poland, Turkey, Switzerland or the United Kingdom (POPRAVKO et al., 1983; GREENAWAY et al., 1989; BANKOVA et al., 1989, 2002, 2006; MARCUCCI, 1995; SALATINO et al., 2011). Related poplar species were *Populus nigra* L. ssp. *nigra*, *P. nigra* L. 'Italica', *P. tremula* L., *P. x canadensis* Moench, *P. alba* L., *and P. tremuloides* Michx. (GREENAWAY et al., 1987; BANKOVA et al., 1989, 1992, 2002; SALATINO et al., 2011). In addition, there have also been reports about *Betula pendula* Roth (MARCUCCI, 1995; BANKOVA et al., 2000), *Acacia* ssp. Mill., *Aesculus hippocastanum* L., *Alnus glutinosa* L., *Pinus* ssp. L., *Prunus* ssp. L. (MARCUCCI, 1995), *Salix alba* L. (SILICI and KUTLUCA, 2005) and presumably *Araucaria* ssp. Juss., *Cupressus sempervirens* L., *Juniperus phoenicea* L., *Platanus x acerifolia* (Aiton) Willd. (BANKOVA et al., 2006) and *Castanea sativa* Mill. (MOREIRA et al., 2008) being potential sources for European propolis.

Poplar trees are also known to be a source for propolis in other parts of the world, such as Algeria, Australasia, North America and Mexico (MARCUCCI, 1995; BANKOVA et al., 2000; CHRISTOV et al., 2006; SALATINO et al., 2011). In addition, non-European plant species such as *Macranga* ssp. (Japan, Taiwan), *Xanthorrhoea* ssp. (Australia), *Plumeria acuminata* W. T. Aiton and *P. acutifolia* Poir. (Hawaii), *Myroxylon balsamum* (L.) Harms and certain conifers (El Salvador) as well as *Ambrosia deltoidea* (Torr.) Payne (Mexico) may serve as further sources for bee glue (MARCUCCI, 1995; WOLLENWEBER and BUCHMANN, 1997; BANKOVA et al., 2006; SALATINO et al., 2011).

Whereas detailed information on the green propolis from Brazil is available demonstrating this material to be collected from *Baccharis dracunculifolia* D.C. (KUMAZAWA et al., 2003; PARK et al., 2004), there is scant literature available on German propolis (HEGAZI et al., 2000; KUNZ et al., 2011; SCHOLL, 2011; CHERNETSOVA et al., 2012), its sources, composition and characteristics. Moreover, only scattered reports are available on the bioactivity of propolis, such as for Dutch (BANSKOTA et al., 2000, 2002) as well as Austrian and French provenances (HEGAZI et al., 2000).

Knowledge of the botanical origin and composition of propolis is of utmost importance for quality control in industry because the compound profile is closely related to the corresponding biological effects. Due to the complex nature of propolis, however, comprehensive identification of all components is hardly possible, and

analytical tools for differentiation are highly desirable. Flavonoids and phenolic acids represent the most active constituents of propolis (LAHOUEL et al., 2010) and appear to be valuable marker substances as their analysis is generally well introduced in quality control laboratories.

Phenolic compounds can be analyzed via thin layer chromatography (TLC), an analytical method amply used for the separation of plant-derived substances because of its suitability for rapid, cost-efficient qualitative analysis and its versatility with respect to detection.

Based on previous work characterizing propolis samples by high performance thin layer chromatography (HPTLC) (SCHOLL, 2011), the differentiation of fingerprints with orange bands (O-type), mainly bluish bands (B-type) (KUNZ et al., 2011) and a third, rather rare fingerprint with additional green bands (G-type) was proposed (SCHOLL, 2011).

The aim of the present work was to analyze a representative set of propolis samples in more detail using TLC and TLC-MS methods. In addition, the propolis samples were to be compared with bud extracts from a range of plant species to identify major botanical sources for German propolis. Finally, the antioxidant properties of individual propolis constituents were to be investigated using DPPH for detection after TLC separation.

Materials and methods

Chemicals and reagents

All chemicals were of analytical grade, and the purity of reference standards was > 95 % unless specified otherwise. Ethanol, ethyl acetate, glacial acetic acid, hydrochloric acid (37 %), methanol, n-hexane, polyethylene glycol 400 (PEG, f.s.), silica gel glass plates (TLC Silica Gel 60, 20 x 20 cm), and silica gel with indicator (orange gel) were from Merck (Darmstadt, Germany). Diphenylboryloxyethylamin (Naturstoffreagenz A, NST), apigenin (Rotichrom® TLC), chrysin, galangin, and naringenin were purchased from Roth (Karlsruhe, Germany). Methanol of CHROMASOLV LC-MS quality, caffeic acid, caffeic acid phenethyl ester (CAPE), kaempferol, pinocembrin and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were from Sigma-Aldrich (Steinheim, Germany). Quercetin was obtained from the United States Pharmacopeial Convention (Rockville, USA), and Acrodisc® syringe filters with GHP membrane (0.45 µm) were purchased from Pall (Dreieich, Germany).

Sampling and storage of propolis and plant exudates

Propolis samples were collected by the Apicultural State Institute of Hohenheim University in 2010 (September/October) and came from six different certified Demeter apiaries located in Central and Southern Germany.

Plants considered for sampling were selected based on literature data of potential propolis sources as well as an obvious stickiness or odor of the buds at the time of collection. Buds from *Populus nigra* L. ssp. *nigra*, *P. nigra* L. 'Italica', *P. tremula* L., *P. x canadensis* Moench 'Robusta', *P. x canescens* (Aiton) Sm., *P. x berolinensis* (K. Koch) Dippel, *P. maximowiczii* A. Henry, *P. balsamifera* L., *Aesculus hippocastanum* L., *Betula pendula* Roth and *B. populifolia* Marsh. were collected from trees in the botanical garden of Hohenheim University (Stuttgart, Germany). Material from *P. alba* L. was collected in Bad Boll, Germany. Harvest time for all bud samples was winter 2012 (January/February). All samples were stored at room temperature until analysis.

Preparation of extracts

Propolis samples were cooled to - 25 °C, ground in an electrical mill under constant water cooling, and passed through a sieve with an aperture of 710 µm. Amounts of 100 mg of propolis were extracted in a round bottom flask with 4 mL of boiling ethanol (water bath, 80 °C ± 3 °C, air cooler) for 15 minutes. The suspension was centrifuged (3000 *g*, 10 min, 10 °C), the supernatant evaporated to dryness, and the residue dissolved in 5 mL ethyl acetate. The obtained solution was filtered through a 0.45 µm membrane syringe filter.

Accordingly, the buds (0.25 - 1.60 g) were extracted with boiling ethanol (ca. 1/5, w/v). After removing the solid plant material, the extract was evaporated to dryness, dissolved in 5 mL ethyl acetate and filtered (0.45 µm).

All extracts were stored at - 25 °C until analysis.

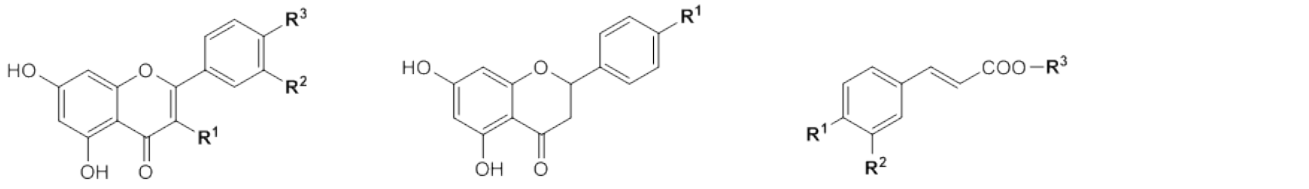
TLC analysis

On the basis of an HPTLC method established earlier (SCHOLL, 2011), a modified TLC method was developed for TLC-MS analyses. With TLC instead of HPTLC, higher sample volumes can be applied on the plate and wider compound bands are achieved, which is advantageous for the subsequent compound elution from the TLC plate using a TLC-MS interface.

TLC plates for MS experiments were washed with methanol (LC-MS quality), dried in a warm air flow, and stored in a clean empty chamber prior to use.

The extracts were applied onto the plate using an Automatic TLC Sampler 4 (CAMAG, Muttenz, Switzerland). Volumes and sample concentrations were varied according to the needs of the detection method (cf. text and figures for details).

A mixture of typical propolis constituents dissolved in methanol



Chrysin (R^1, R^2, R^3 = H)
Apigenin (R^1, R^2 = H, R^3 = OH)
Galangin (R^1 = OH, R^2, R^3 = H)
Kaempferol (R^1, R^3 = OH, R^2 = H)
Quercetin (R^1, R^2, R^3 = OH)

Pinocembrin (R^1 = H)
Naringenin (R^1 = OH)

Caffeic acid (R^1, R^2 = OH, R^3 = H)
CAPE (R^1, R^2 = OH, R^3 = $CH_2CH_2C_6H_5$)

Fig. 1: Structure of the reference compounds.

(apigenin, CAPE, kaempferol, quercetin (each 0.63 µg/mL), caffeic acid (0.31 µg/mL), chrysin (1.88 µg/mL), galangin (0.94 µg/mL), naringenin (5.63 µg/mL), and pinocembrin (2.50 µg/mL) was analyzed as reference (Fig. 1). After sample application, the plates were dried in a chamber with approximately 10 % relative humidity for at least 1.5 h.

The mobile phase consisted of hexane / ethyl acetate / glacial acetic acid (5:3:1, v/v/v). The second trough of the twin trough chamber contained a filter paper (20 x 20 cm) wetted with 10 mL of concentrated hydrochloric acid. After developing the plates in the unsaturated twin trough chamber they were dried for about 5 min in a warm air flow.

Phenolic compounds were derivatized by spraying with 1 % NST in methanol (Neu's reagent) and 5 % PEG in methanol and visualized 30 min after spraying under ultraviolet light (366 nm) using a PC-controlled documentation system (ProViDoc system VD60, Camera Oscar F-320IC, biostep, Jahnsdorf, Germany).

To evaluate the antioxidant potential of the extracts, the plates were sprayed with 0.2 % DPPH reagent in methanol and kept at room temperature until optimum coloration. Antioxidant components appeared as yellow spots against a purple background.

TLC-UV/MS conditions

TLC-MS analyses were carried out using a CAMAG TLC-MS-Interface (elution head oval, 4 × 2 mm, Muttenz, Switzerland). The interface was connected between an HPLC pump and a diode array detector (DAD) connected in series to an MS detector. The eluent flow was generated by an Agilent Technologies (Waldbronn, Germany) 1200 HPLC system consisting of a vacuum degasser (G1379B), a binary pump (B1312A), an autosampler (G1329A) and a DAD (G1315B). Pure methanol was used as mobile phase at a flow rate of 0.1 mL/min. UV/Vis data were recorded using Agilent ChemStation (Rev. B.01.03). DAD acquisitions were performed in a range of 210 - 500 nm, and the signal was monitored at 280 nm. The DAD was coupled with a Bruker Daltonics (Bremen, Germany) HCTultra ion trap equipped with an electrospray ionization (ESI) interface. MS data were recorded using EsquireControl (Version 6.1) and processed with Bruker DataAnalysis (Version 3.4).

Mass spectrometric analyses were carried out in negative ionization mode with a scan range of *m/z* 50 - 1000. ESI parameters were set as follows: the capillary voltage was 4000 V with a target mass-to-charge ratio of 200, the nebulizer (N_2) pressure was set at 30 psi, the drying gas (N_2) temperature at 325 °C and the drying gas flow rate at 7 L/min.

Results and discussion

Comparison of propolis types with bud extracts by TLC

Previous investigations have shown that German propolis samples may be divided into different groups depending on the TLC fingerprints of their phenolic fractions (NST/PEG-reagent) (Kunz et al., 2011; Scholl, 2011). In the present study, 13 propolis samples were investigated, six of which were assigned to the O-type, six to the B-type and one to the G-type. The so-called O-type (Fig. 2A) was predominant and showed several orange bands (typical for flavonoids like quercetin) apart from distinct light blue and some green bands. The B-type (Fig. 2B) was characterized by weak orange and light green bands or their absence, whereas light and dark blue bands were characteristic colors which are typical for caffeic acid and *p*-coumaroyl or feruloyl derivatives. The G-type (Fig. 2C) was rare and exhibited bands similar to those of the O-type, but of lower intensity, in addition to a weak green and one or two stronger green bands.

The comparison of the TLC profiles with those of ethanolic bud extracts indicated the main sources for O-type propolis to be *Populus x canadensis* and *P. nigra* 'Italica' (Fig. 3A). *Populus nigra* ssp. *nigra*, however, is an endangered species in Germany (Roloff, 2006). Hence, cultivated hybrids are assumed to play a more important role.

Bud extracts from *P. x berolinensis, P. maximowiczii,* and *P. balsamifera* also showed typical bands characteristic of black poplar (Fig. 4). It is interesting to note that the light blue bands of caffeates (R_f 0.4 - 0.6) were absent in *P. balsamifera* extracts, thus confirming the results of Isidorov et al. (2003). As the latter three species rarely occur in Germany, they were not considered further in the present work.

The bud extract from *P. tremula* (Fig. 3B) revealed two light blue bands (R_f ~ 0.13 and 0.19) which were also observed in B- and some O-type propolis samples. One of these bands (R_f 0.19) also appeared to be a constituent of *P. x canadensis* (Fig. 3A), whereas the second (R_f 0.13) was only detected in *P. tremula* (Fig. 3B). This finding indicates *P. tremula* to be a potential plant source for B-type propolis. The chromatographic profiles of *P. alba* and *P. x canescens* did not show further strong coherence with that of B-type propolis.

The identification of potential G-type propolis plant sources was challenging because of partial overlap with poplar hybrid bands. The TLC data suggested similarities of green bands (R_f 0.45 and 0.65) detected in propolis samples with those observed in *A. hippocastanum*, *B. pendula* (Fig. 5C) and *B. populifolia* extracts, respectively (Fig. 4).

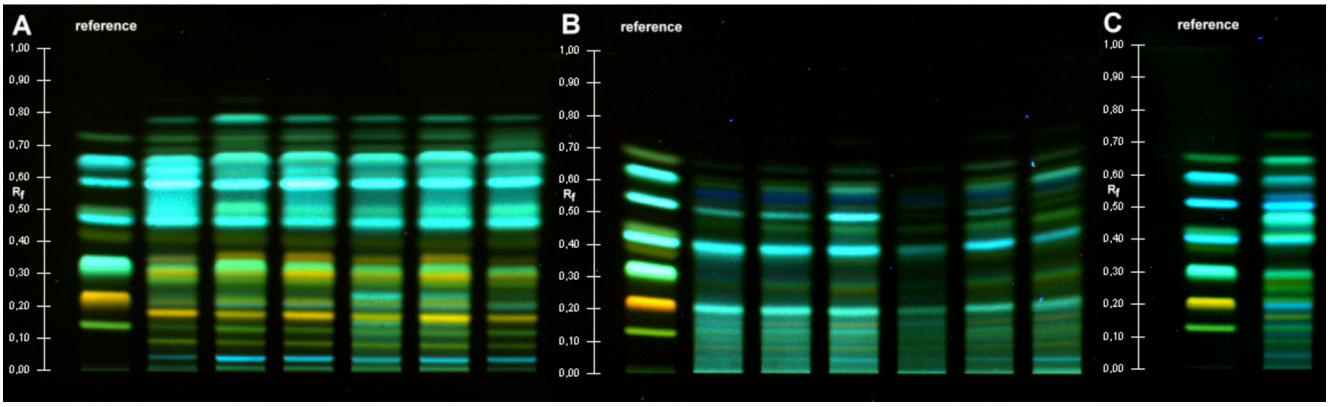


Fig. 2: TLC fingerprints of (A) O-type propolis samples with characteristic light blue, green and yellow bands, (B) of B-type samples with light and dark blue colors and (C) of G-type propolis with additional green bands. Applied volume for each propolis sample was 10 µL and 25 µL for the reference mixture, respectively.

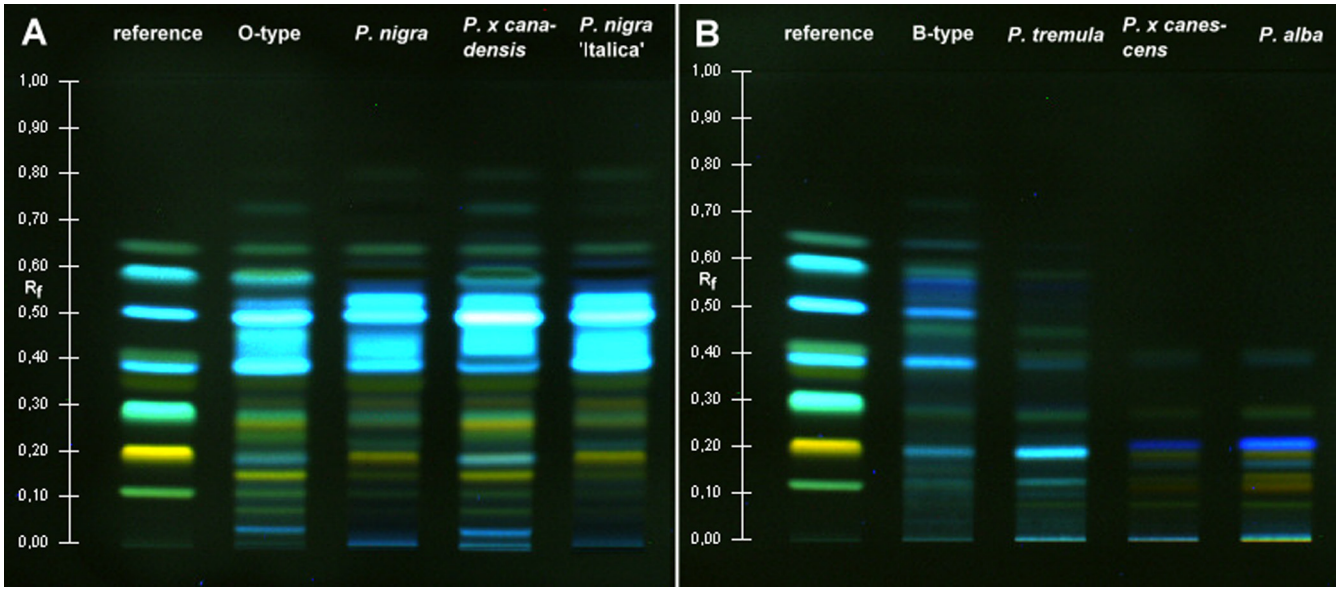


Fig. 3: TLC fingerprints of bud extracts from (A) black poplars (each 10 µL) and (B) *P. tremula* L. (20 µL), *P. x canescens* (Aiton) Sm. and *P. alba* L. (each 30 µL) in comparison to propolis of O- and B-type, respectively (10 µL).

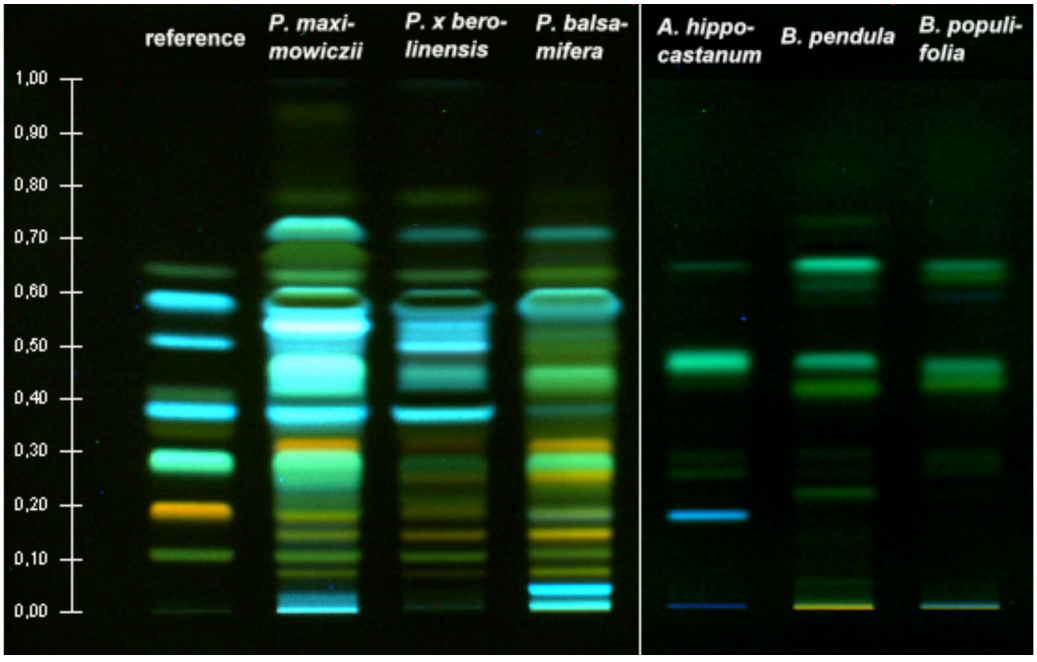


Fig. 4: TLC fingerprints of bud extracts from *Populus maximowiczii* A. Henry, *P. x berolinensis* (K. Koch) Dippel, *P. balsamifera* L., *Aesculus hippocastanum* L., *Betula pendula* Roth and *B. populifolia* Marsh. (each 10 µL). The applied volume for the reference was 25 µL.

More detailed investigations of these similarities were performed by comparison of some of the bands via TLC-MS analysis and by TLC autography with DPPH.

Detailed characterization of the phenolic profile by TLC-MS experiments

Propolis is a complex mixture, and more than 300 constituents have been described (Banskota et al., 2001b). Consequently, the identification of individual propolis compounds by TLC-MS is demanding due to co-elution phenomena. This becomes particularly evident when TLC zones are analyzed by mass spectrometry. Negative ion ESI-MS spectra of the propolis extracts often indicated more than one compound to be present, which was also reflected by the UV/Vis spectra, suggesting coelution. Thus, compound assignment and structure characterization was challenging.

Based on TLC fingerprints, the propolis samples were compared to the bud extracts of potential plant sources. Identification of individual compounds was achieved by comparison of UV/Vis spectra and MS data with authentic references and literature data.

Detailed results of UV spectroscopic and mass spectrometric analyses of the reference standards (Fig. 5A) are summarized in Tab. 1.

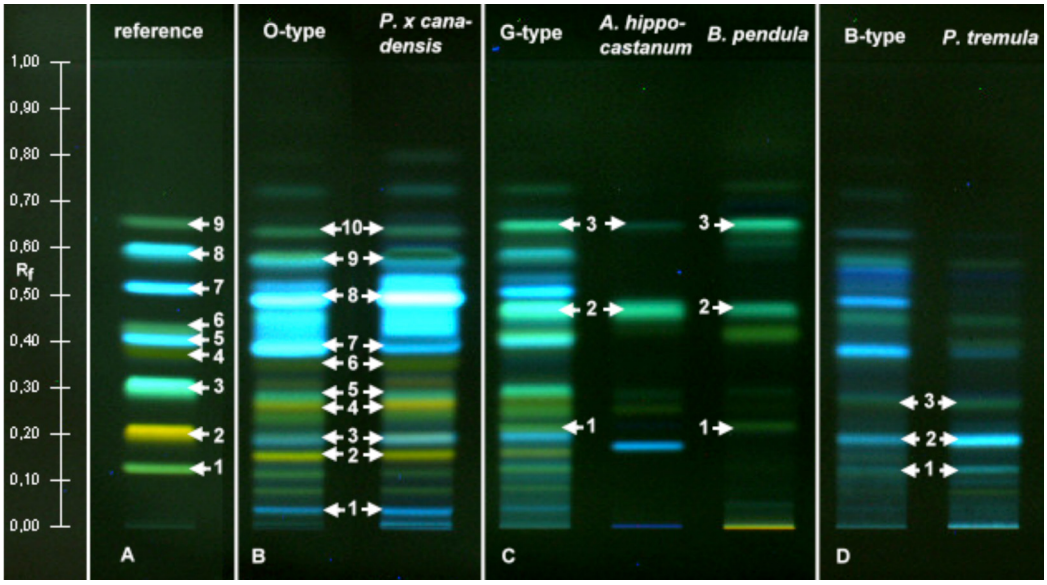


Fig. 5: Chromatographic separation specifying the bands investigated in TLC-MS experiments. (A) Reference mixture, (B) O-type propolis and *P. x canadensis* bud extracts, (C) G-type propolis and *A. hippocastanum* L. and *B. pendula* Roth bud extracts, (D) B-type propolis and *P. tremula* L. bud extract.

Tab. 1: TLC-MS and UV spectroscopic data obtained from the analysis of a reference mixture (see Fig. 1, 5A)

Band	Compound	Band color	UV_{max} [nm]	$[M-H]^-$ [*m/z*]	MS^2 experiment[a] [*m/z*]	MS^3 experiment[a,b] [*m/z*]
1	Apigenin	green	268, 336	269[c]	-[e]	-[e]
2	Quercetin	orange	294, 366[d]	301	179, 151, 107	151, 169, 107
3	Kaempferol	green	268, 294, 368[d]	285	151, 257, 211, 169	169
4	Chrysin	green-brown	270, 324sh	253	209, 253, 151, 165	153, 181
5	Caffeic acid	light blue	274[d]	177[d]	135, 105, 147	-[e]
6	Naringenin	green	288	271	151, 211, 177, 107	107, 169
7	CAPE	light blue	296, 324	283	179, 135	135
8	Galangin	light blue	268, 292, 356[d]	269[c]	-[e]	-[e]
9	Pinocembrin	green	288	255	213, 151, 187	183, 211, 169, 133

a: fragment ions are listed according to their signal intensity in decreasing order
b: fragments released from base peak signals in MS^2 experiments
c: interferences with masses from the TLC plate, no fragmentation data available
d: concentration of compound too low for UV and/or MS detection and/or coelution
e: not detectable
sh: shoulder

An O-type propolis sample (10 µL) was compared to an extract of *P. x canadensis* buds (10 µL), and 10 bands matching in color were investigated more closely (Fig. 5B). The corresponding data are listed in Tab. 2 and 3. The MS and UV/Vis data of band 1 of both samples coincided, and this compound was assigned to methoxy-chrysin. Two orange bands (2 and 4) of the poplar extract were identified as quercetin derivatives. Quercetin-methyl-ether (band 2) produced an ion at *m/z* 315 and a fragment at *m/z* 300 in the MS^2 experiment due to the loss of a methyl radical ($[M-H-CH_3]^-$) as well as fragments at *m/z* 271 ($[M-H-CO_2]^-$) and *m/z* 256 ($[M-H-CH_3-CO_2]^-$) in the MS^3 experiment. The compound of band 4 produced an ion at *m/z* 329, showing two losses of each 15 Da in the MS^2 experiment resulting in fragment ions at *m/z* 314 and 299, respectively. This indicates the loss of two methyl groups, and the compound was therefore tentatively identified as quercetin-dimethyl-ether. Quercetin-methyl-ether (band 2) was also found in the O-type propolis sample together with kaempferol-methyl-ether, which produced an ion at *m/z* 299, whereas quercetin-dimethyl-ether was not detected in band 4 of the propolis extract. Both samples revealed the presence of two further compounds in band 4 which yielded one ion at *m/z* 299, tentatively identified as luteolin-methyl-ether, and one unidentified ion at *m/z* 283. Band 3 of both propolis and *P. x canadensis* bud extracts was identified as pinobanksin-methyl-ether with a product ion at *m/z* 285 and fragments in the MS^2 experiment

Tab. 2: TLC-MS and UV spectroscopic data obtained from the analysis of a *Populus* x *canadensis* Moench 'Robusta' bud extract (see Fig. 5B)

Band	Compound	Band color	UV_{max} [nm]	$[M-H]^-$ [*m/z*]	MS^2 experiment[a] [*m/z*]	MS^3 experiment[a,b] [*m/z*]
1	Chrysin-methyl-ether[e]	blue	288, 350sh[d]	283	268, 239, 211	239, 211
2	Quercetin-methyl-ether[e]	orange	288, 338[d]	315	300	271, 255
	Kaempferol-methyl-ether[e]			299	284, 271, 255	284, 257
3	Pinobanksin-methyl-ether[e]	blue	288	285	267, 252, 239, 224	252, 224
4	Luteolin-methyl-ether[e]	yellow	290, 344[d]	299	284	255, 227
	Quercetin-dimethyl-ether[e]			329	314	299, 285, 271, 243
	Unidentified			283	268, 255	252, 240, 224
5	Quercetin-methyl-ether[e]	green	290, 346[d]	315	300, 256, 193, 165	271, 256, 151
	Kaempferol			285	151, 283, 257, 165	169, 107
6	Chrysin	green-brown	272, 320	253	181, 209, 253, 165	139
7	Caffeic acid	light blue	296, 332[d]	177[d]	135, 89	105
8	Caffeic acid benzyl ester (CABE)[e]	light blue	238, 298sh, 328[d]	269	178, 134, 161, 225	134
	CAPE			283	179, 135, 161	135
	Caffeic acid cinnamoyl ester[e]			295	178, 134, 211, 251	134
9	Galangin	blue green	294, 318[d]	269[c]	-[f]	-[f]
	Pinobanksin-acetate[e]			313	253, 271	209, 180, 107
10	Pinocembrin	slightly green	290[d]	255	213, 151, 187	183, 169, 133
	Pinobanksin-butyrate[e]			341	253, 271	209, 253, 181, 151
	Methylbutyroyl-pinobanksin[e]			355	253, 271	209, 253, 181

a: fragment ions are listed according to their signal intensity in decreasing order
b: fragments released from base peak signals in MS^2 experiments
c: interferences with masses from the TLC plate, no fragmentation data available
d: concentration too low for UV and/or MS detection and/or coelution
e: tentatively identified by comparison with literature data (Banskota et al., 2002; Usia et al., 2002; Gardana et al., 2007; Pellati et al., 2011; Shi et al., 2012)
f: not detectable
sh: shoulder

at *m/z* 267 ($[M-H-H_2O]^-$), *m/z* 252 ($[M-H-H_2O-CH_3]^-$), *m/z* 239 ($[M-H-H_2O-CO]^-$), *m/z* 224 ($[M-H-H_2O-CH_3-CO]^-$), and at *m/z* 252 and 224 in the MS^3 experiment, respectively.

Band 5 proved to be a coelution of kaempferol and quercetin-methyl-ether, while band 6 was identified as chrysin. Band 7 only showed a weak signal at *m/z* 177, although it appeared as a strong band on the TLC plate. This compound was assigned to caffeic acid based on its chromatographic behavior. Interestingly, the mass spectrometric characteristics were atypical for caffeic acid revealing a predominant product ion at *m/z* 177, which is presumably due to the low substance amounts and interference with matrix compounds of the TLC plate. The identity of caffeic acid was finally corroborated with a reference compound showing an identical mass spectrometric pattern.

CAPE produced an $[M-H]^-$ ion at *m/z* 283, CABE (caffeic acid benzyl ester) at *m/z* 269 and caffeic acid cinnamoyl ester at *m/z* 295. These three compounds coeluted as a light blue-colored zone (band 8) in both extracts. Band 9 was identified as galangin with a product ion at *m/z* 269 and pinobanksin-acetate at *m/z* 313. The compounds of band 10 revealed a close match with the MS and UV/Vis spectral data of pinocembrin, pinobanksin-butyrate and methylbutyroyl-pinobanksin coeluting with the two aforementioned compounds (Fig. 5B).

The green bands of G-type propolis samples (20 µL) were compared to the TLC patterns of *B. pendula* (20 µL) and *A. hippocastanum* (30 µL; Fig. 5C) bud extracts. Both *B. pendula* and propolis samples revealed the presence of a compound at *m/z* 283 which was tentatively assigned to apigenin-methyl-ether or galangin-methyl-ether (band 1). Band 1 of the propolis sample further revealed ions at *m/z* 441 and 471, which were assigned to acetyl-*p*-coumaroyl-caffeoylglycerol and acetyl-caffeoyl-feruloylglycerol. These two compounds were also detected in *P. tremula* extracts. Band 2 showing green color revealed an ion at *m/z* 299 in all three samples, however, the corresponding compounds detected in *B. pendula* and *A. hippocastanum* extracts differed in their fragmentation behavior. The compound detected in propolis and *B. pendula* showed ions at *m/z* 284 and 165 in the MS^2 experiment and at *m/z* 151 as base peak in the MS^3 experiment, whereas the compound detected in *A. hippocastanum* revealed highest intensities at *m/z* 165, 271 and 284 in the MS^2 and *m/z* 121 in the MS^3 experiment. The ion at *m/z* 299 might be assigned to kaempferol derivatives, e.g. kaempferide or rhamnocitrin, and the loss of 15 Da indicates the release of a methyl group from an aromatic methoxy function upon collision-induced dissociation (CID). While the UV/Vis spectra were also in good agreement with those

Tab. 3: TLC-MS and UV spectroscopic data obtained from the analysis of O-type propolis (see Fig. 5B)

Band	Compound	Band color	UV_{max} [nm]	$[M-H]^-$ [*m/z*]	MS^2 experiment[a] [*m/z*]	MS^3 experiment[a,b] [*m/z*]
1	Chrysin-methyl-ether[e]	blue	286[d]	283	268, 239, 211, 168	239, 211
2	Quercetin-methyl-ether[e]	orange	288[d]	315	300, 256	271, 255
	Kaempferol-methyl-ether[e]			299	284, 271, 255	284, 256, 227, 200
	Unidentified			349	334	298, 270, 242
3	Pinobanksin-methyl-ether[e]	blue	288	285	267, 283, 252, 239	252, 224
4	Luteolin-methyl-ether[e]			299	284	255, 227
	Unidentified			283	268	240, 268, 172
5	Quercetin-methyl-ether[e]			315	300, 257	272, 151
	Kaempferol			285	151, 283, 199, 257	107
6	Chrysin	green-brown	282, 334sh	253	209, 253, 181	180, 143, 165
7	Caffeic acid	light blue	290, 330[d]	177[d]	135, 133	-[f]
8	Caffeic acid benzyl ester (CABE)[e]			269	178, 134, 210, 161	152
	CAPE			283	179, 135	135
	Caffeic acid cinnamoyl ester[e]			295	178, 134, 251, 211	134
9	Galangin			269[c]	-[f]	-[f]
	Pinobanksin-acetate[e]			313	253, 271	209, 181, 226
10	Pinocembrin			255	213, 253, 151	183, 211
	Pinobanksin-butyrate[e]			341	253	209, 253
	Methylbutyroyl-pinobanksin[e]			355	253, 271	209, 180, 225

a: fragment ions are listed according to their signal intensity in decreasing order
b: fragments released from base peak signals in MS^2 experiments
c: interferences with masses from the TLC plate, no fragmentation data available
d: concentration too low for UV and/or MS detection and/or coelution
e: tentatively identified by comparison with literature data (Banskota et al., 2002; Usia et al., 2002; Gardana et al., 2007; Pellati et al., 2011; Shi et al., 2012)
f: not detectable
sh: shoulder

of a kaempferol derivative, mass spectrometric analysis of band 3 did not allow close structural assignment even though color and R_f values were quite similar. In the *A. hippocastanum* extract, a strong blue band appeared that was tentatively identified as esculetin, a specific horse chestnut constituent. As this band was completely missing in the propolis samples, *A. hippocastanum* was concluded to be of minor importance as a propolis source. This was somewhat surprising as horse chestnut is characterized by very sticky buds rich in exudate and is fairly abundant in Germany.

Three bands of the B-type sample (30 µL) were compared to *P. tremula* (40 µL; Fig. 5D, see Tab. 4 and 5 for detailed results). Band 1 was identified as acetyl-dicaffeoylglycerol producing an ion at *m/z* 457 which was present in both samples. Upon CID, fragments at *m/z* 161 and 135 were observed indicating the presence of caffeic acid. Acetyl-*p*-coumaroyl-caffeoylglycerol exhibiting an $[M-H]^-$ ion at *m/z* 441 and acetyl-feruloyl-caffeoylglycerol at *m/z* 471 were found for the light blue band 2. For those substances, chlorine adducts at *m/z* 477 and 507, respectively, were also observed, which were due to the solvents applied for TLC development. Band 3 was found to contain acetyl-*p*-coumaroyl-feruloylglycerol at *m/z* 455 and acetyl-di-*p*-coumaroylglycerol at *m/z* 425, known constituents of aspen buds (Popracko et al., 1983), which were verified by their characteristic fragmentation behavior. Again, chlorine adducts were observed upon mass spectrometric analyses.

Greenaway et al. (1989) as well as Klimczak et al. (1972) found the phenolic acid composition of poplar buds to remain stable during the vegetation period. In addition, there is so far no evidence that individual propolis components are chemically modified by bee enzymes (Bankova et al., 2006). Our results support this finding as there was surprisingly good agreement between the fingerprints of O-type and B-type propolis and poplar bud extracts. It is quite likely that e.g. black poplar hybrid buds are an attractive propolis source for bees because these buds generally are highly sticky and spread an aromatic odor. Bees obviously take advantage of the substantial amount of bioactive exudates produced by this poplar species.

Investigations into the antioxidant potential by TLC-DPPH analysis

The radical scavenging activity of propolis has been well documented (Kumazawa et al., 2004; Moreira et al., 2008; Yang et al., 2011). Most authors have determined the antioxidant potential of propolis extracts using photometric assays. However, this approach does not allow determining the contribution of individual compounds to the

Tab. 4: TLC-MS and UV spectroscopic data obtained for the analysis of *P. tremula* L. (see Fig. 5D)

Band	Compound	Band color	UV_{max} [nm]	$[M+Cl]^-$ [*m/z*]	$[M-H]^-$ [*m/z*]	MS^2 experiment[a] [*m/z*]	MS^3 experiment[a,b] [*m/z*]
1	Acetyl-dicaffeoylglycerol[d]	slightly light blue green	292, 318[c]	-[e]	457	295, 397, 161, 235, 135	235, 161
	Unidentified			587	551	429, 323, 491, 191	323, 137, 387
2	Acetyl-*p*-coumaroyl-caffeoylglycerol[d]	light blue green	320[c]	477	441	381, 295, 179, 235, 135	179, 163, 135
	Acetyl-feruloyl-caffeoylglycerol[d]			507	471	411, 193, 295, 179	193, 179, 135
3	Acetyl-*p*-coumaroyl-feruloylglycerol[d]	dark blue	318[c]	491	455	395, 193, 163, 134	193, 163, 351, 134
	Acetyl-di-*p*-coumaroylglycerol[d]			461	425	365, 163, 321, 215	163, 321, 215, 119

a: fragment ions are listed according to their signal intensity in decreasing order
b: fragments released from base peak signals in MS^2 experiments
c: concentration too low for UV and/or MS detection and/or coelution
d: tentatively identified by comparison with literature data (Banskota et al., 2002; Usia et al., 2002; Gardana et al., 2007; Pellati et al., 2011; Shi et al., 2012)
e: not detectable

Tab. 5: TLC-MS and UV spectroscopic data obtained from the analysis of B-type propolis (see Fig. 5D)

Band	Compound	Band color	UV_{max} [nm]	$[M+Cl]^-$ [*m/z*]	$[M-H]^-$ [*m/z*]	MS^2 experiment[a] [*m/z*]	MS^3 experiment[a,b] [*m/z*]
1	Acetyl-dicaffeoylglycerol[d]	light blue green	288, 318sh	-[e]	457	295, 397, 235, 161	235, 161
2	Acetyl-*p*-coumaroyl-caffeoylglycerol[d]	light blue green	292, 318[c]	477	441	381, 295, 179, 163	179, 163, 135
	Acetyl-feruloyl-caffeoylglycerol[d]			507	471	411, 193, 295	193, 179, 135
3	Acetyl-*p*-coumaroyl-feruloylglycerol[d]	dark blue	318[c]	491	455	395, 193, 163, 179	193, 163, 351, 134
	Acetyl-di-*p*-coumaroylglycerol[d]			461	425	365, 163, 321, 215	163, 321, 215, 119

a: fragment ions are listed according to their signal intensity in decreasing order
b: fragments released from base peak signals in MS^2 experiments
c: concentration too low for UV and/or MS detection and/or coelution
d: tentatively identified by comparison with literature data (Banskota et al., 2002; Usia et al., 2002; Gardana et al., 2007; Pellati et al., 2011; Shi et al., 2012)
e: not detectable
sh: shoulder

overall activity. Jasprica et al. (2007) have demonstrated TLC in combination with the DPPH assay to be a reliable method for the analysis of antioxidant capacity in propolis. Consequently, this system was also applied in the present work.

A range of antioxidants was detected in all propolis types. Applying a sample volume of 20 µL, the O-type propolis displayed greatest reducing capacity, in particular in the R_f range of 0.4 to 0.7 (Fig. 6A). This finding is well in accordance with the TLC-MS results revealing that well-known antioxidants such as caffeic acid, CAPE and their derivatives, chrysin, galangin, naringenin and pinocembrin were present in this R_f range.

G- and B-type propolis extracts showed lower antioxidant activity. Active compounds were also clearly detectable in the bud extracts of *P. nigra*, *P. x canadensis* and *P. nigra* 'Italica' (each 20 µL). The bud extract of the black poplar hybrid *P. x canadensis* showed best agreement of the DPPH fingerprint with that of the O-type propolis extract (Fig. 6B).

Extracts from *B. pendula*, *B. populifolia* and *A. hippocastanum* (each 30 µL) (Fig. 7A) as well as *P. tremula* and *P. x canescens* (each 40 µL) (Fig. 7B) displayed weaker or no DPPH activity. The light blue bands of *P. tremula* (NST/PEG detection) exhibited weak reducing power applying the DPPH reagent. The same bands were located in B-type propolis thus supporting the assumption that *P. tremula* is an important plant source of this propolis type. Moreover,

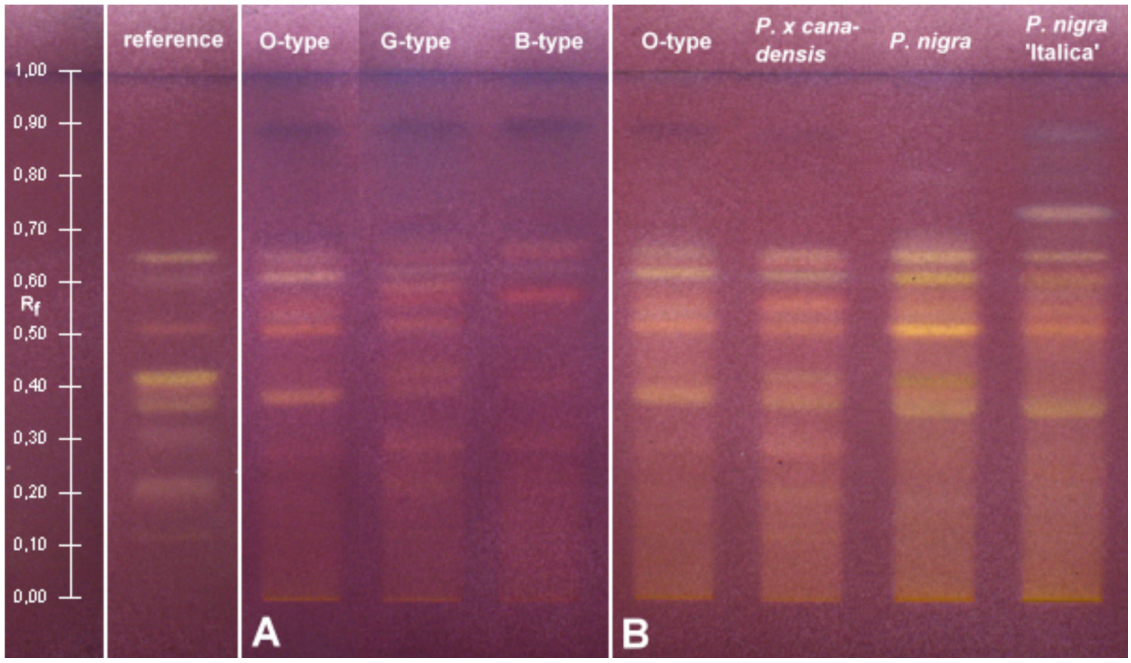


Fig. 6: TLC fingerprints of the DPPH experiment with (A) the three propolis types in comparison and (B) the O-type propolis with *P. nigra* L. *ssp. nigra*, *P. x canadensis* Moench 'Robusta' and *P. nigra* L. 'Italica' bud extracts (each 20 µL). The applied volume for the reference was 40 µL.

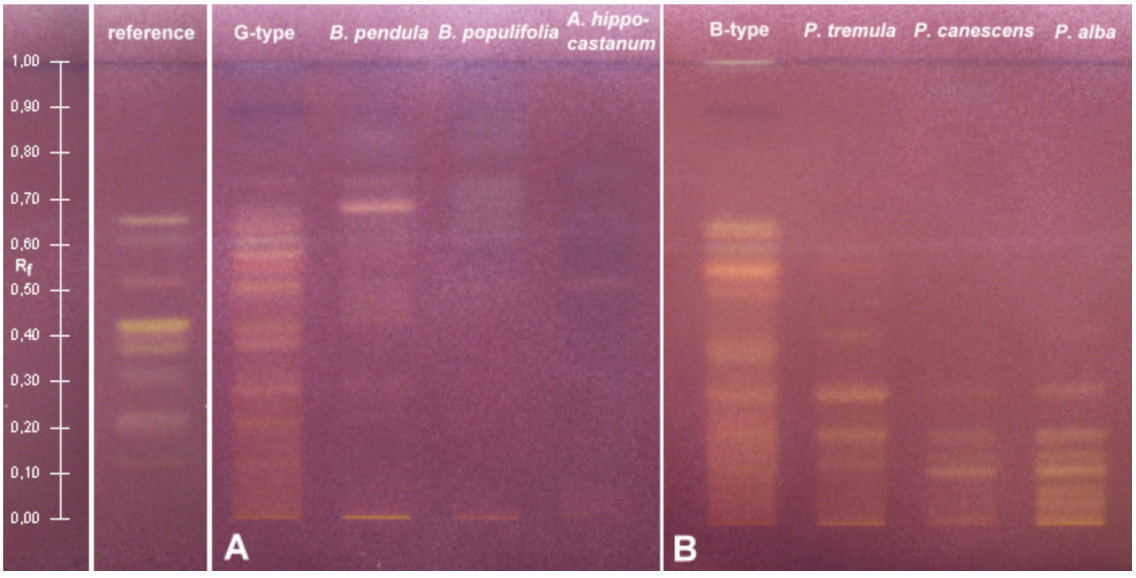


Fig. 7: TLC fingerprints of the DPPH experiment with (A) the G-type propolis with *B. pendula* Roth, *B. populifolia* Marsh. and *A. hippocastanum* L. bud extracts (each 30 µL) and (B) the B-type propolis and with *P. tremula* L., *P. x canescens* (Aiton) Sm. and *P. alba* L. bud extracts (each 40 µL). The applied volume for the reference was 40 µL.

no agreement of DPPH zones with those of G-type propolis was found for *B. pendula*, *B. popufolia* and *A. hippocastanum* extracts. This finding is again in agreement with the TLC-MS results.

Thus, O-type propolis is characterized by a wide range of flavonoids and caffeic acid derivatives and appeared to be the sample type displaying greatest antioxidant potential. However, allergens like CABE and CAPE were also detected in the black poplar propolis type. Both compounds were more abundant in O-type propolis than in B-type samples, which is crucial for quality control of products for topical application.

Conclusion

The developed TLC method is a straightforward and efficient way for screening propolis samples and for quality control purposes. It is applicable to qualitatively determine biologically active compounds like flavonoids and phenolic acids in propolis using the specific detection reagent NST/PEG and to monitor the antioxidant power with DPPH applied as alternative detection reagent. With the help of TLC-MS experiments, we were able to assign more than 15 compounds in both propolis and bud exudates and to find congruence between

the profiles of the bee product and the corresponding plant source. Black poplar hybrids (*P. x canadensis*) as well as aspens (*P. tremula*) appear to be the main sources for bees to collect propolis in Central and Southern Germany. While there is indication that birch (*B. pendula*) is also a potential source, the chemical proof was not as clear as for black poplar hybrids or aspens.

When assessing propolis quality, investigations on the compound profile should always be combined with assays allowing an estimation of the potential with regard to chemical reactivity or bioactivity. Such activity-guided analyses are helpful to select propolis with desired properties such as antioxidant, antibiotic or antiviral activities. Consequently, analytical methods are needed which generate results meaningful with respect to the intended use of propolis in foods, cosmetic or medicinal products.

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