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Function of floral pigments in the orchid genus *Ophrys*

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I. General Introduction

1. The orchid genus *Ophrys*

The orchid genus *Ophrys* became a favoured and well examined example for a plant genus possessing sexually deceptive flowers. This highly selective and specialized way of reproduction was observed to occur among the Orchidaceae exclusively (DAFNI 1984, NILSON 1992).

1.1 Ophrys in the plant kingdom

The genus *Ophrys* L. is part of the second largest plant family among the angiosperms (flowering plants), the cosmopolitan Orchidaceae. All *Ophrys* species are terrestrial. (PRIDGEON et al. 2001).

Spermatophyta (phylum)

Magnoliophytina (subphylum)

Liliopsida (classis)

Liliidae (subclassis)

Orchidales (ordo)

Orchidaceae (familia)

Orchidoideae (subfamilia)

Orchideae (tribus)

Orchidinae (subtribus)

Ophrys (genus)

The tendency of several *Ophrys* species towards natural hybridization generates confusingly high species diversity (EHRENDORFER 1980). Therefore, classification and correct separation within the genus turned out to be quite difficult. (DELFORGE 2005).

1.2 Etymology

The etymological origin of the genus name "*Ophrys*" is ancient Greek and this word means "eyebrow". Gaius Plinius Secundus, better known as Pliny the Elder, was said to be the first one who described a plant with this name in his encyclopedia "Naturalis Historia" (Natural history) written AD 77. Another theory claimed that the name first occurred in Carl von Linné´s "Species Plantarum", 1753. The most proper reason for this name was supposed to be the remarkable labellum with its furry surface (FÜLLER, F., 1982, PRIDGEON et al. 2001).

1.3 Distribution of the genus Ophrys

The genus is considered as one of the most eminent among European orchids. The majority of *Ophrys* species is distributed in the Mediterranean region including South Europe and islands the Aegean Sea, Crete, Sicily, Corsica and Sardinia, islands near the dalmatic coast as well as the Balearic Islands and Cyprus (BENISTON & BENISTON 1999, PAULUS & GACK 1990b). Several species are also native in Central and North Europe (ROTHMALER 2002, VAN DER CINGEL 1995, BAUMANN et al. 2006) and some species, e.g. *O. sphegodes*, are even described from the south of England and others also from North Africa and from Asia Minor to the Caucasus region (DELFORGE 2005).

Four species are indigenous to Austria. The Pannonian climate in the North-East, East, South-East and South favours the occurrence of *Ophrys* (JANCHEN 1977, PERKO 2004). According to their individual flowering times, they comprise *O. sphegodes* (April-May), *O. insectifera* (May-June), *O. holoserica* (May-June) and *O. apifera* (June) (DELFORGE 2005, SVOJTKA 2006; see Fig. 1).

O. apifera Huds.

(Bee orchid)

O. sphegodes Mill.

(Early spider orchid)

O. holoserica (Burm f.) Greuter

(Late spider orchid)

O. insectifera L.

(Fly orchid)

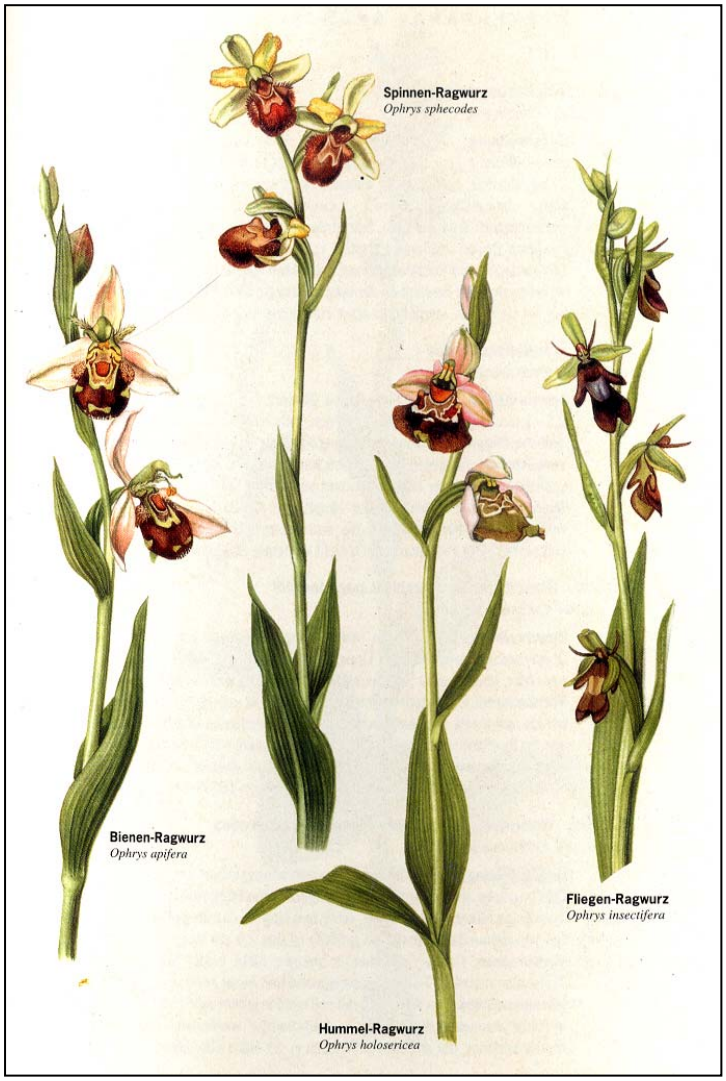

Fig. 1: *Ophrys* species in Austria: from left: *O. apifera*, *O. sphegodes*, *O. holoserica*, *O. insectifera*, (AICHELE & GOLTE-BECHTLE 2005).

1.4 Floral morphology

Three subfamilies characterised by three different floral shapes distinguish the Orchidaceae. The most primitive flower shape is to be found in orchid genera classified within the subfamily Apostasioideae. Belonging to the Orchidoideae the genus *Ophrys* is part of the most advanced orchid subfamiliy (PRIDGEON et al. 2001). The characteristic labellum corresponds to the median tepal of the inner perigone. Extended coloration patters are assumed to be primitive, such as seen in the lips of *O. speculum* and *O. fusca*. The highly complex and individual patterns of species belonging to the O. *holoserica-oestrifera* group (e.g. *O. holoserica*, *O. heldreichii*) are supposed to constitute advanced species instead (PAULUS 2007). All tepals are free. The two tepals of the inner perigone circle, which do not form the labellum, are reduced and small. In contrast, the tepals of the outer perigone are bigger and not modified in any way (SANFORD 1974, SUNDERMANN 1980). Only one stamen of originally six is left, whereas the others are reduced entirely or modified to staminodia (Fig. 2).

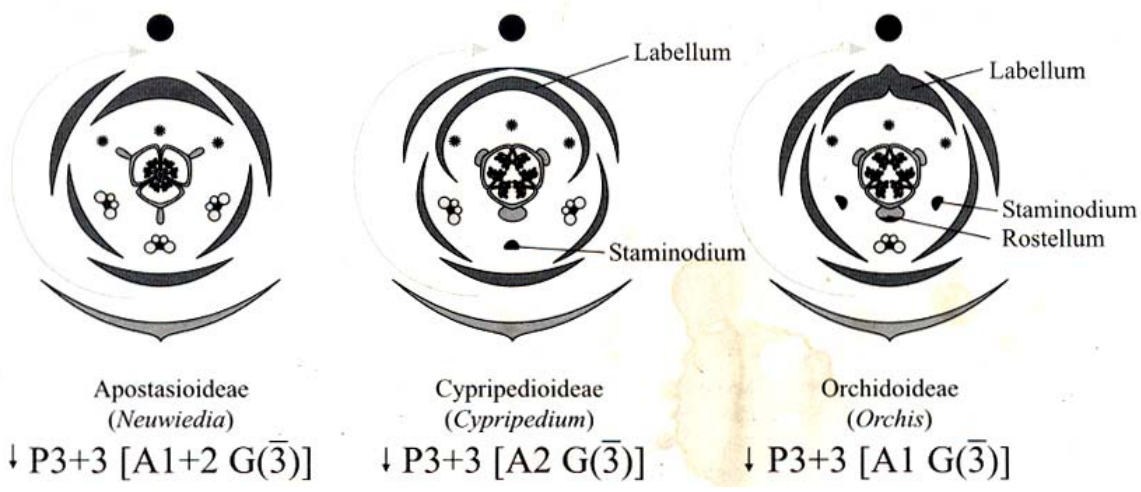

Fig. 2: Floral graphs and foral formulae of the three orchid subfamilies (STÜTZEL 2002)

1.5 Pollination biology: Sexual deception

The complex pollination of *Ophrys* is based on Batesian floral mimicry. In strict sense, this phenomenon depicts the imitation 'mimicking' of a specific model and a response of an operator reacting to the imitation at the same time (SCHLÜTER & SCHIESTL 2008). Males of various insect species are attracted to the flowers of *Ophrys*. However, no floral rewards are offered in return. The pollinating species comprise mostly bees (Andrenidae, Anthophoridae, Colletidae, Megachilidae and Apidae), but also wasps (Sphecidae, Scoliidae) and two beetle species (Blitopertha and Phyllopertha, Scarabaeidae). The latter are not original pollinators but also get attracted to the flowers occasionally (KULLENBERG 1961, BORG-KARLSON 1990, PAULUS & GACK 1990a, PAULUS 1997, 2007).

Ophrys flowers produce a complex flower odour in order to feign a female ready for mating. The olfactory signals initiate sexual behaviour in the male visitors, which are trying to copulate with the labellum. During pseudocopulation, the pollinaria get attached to the pollinators and are transferred if another *Ophrys* flower gets visited (PAULUS 1997, SCHIESTEL et al. 1999, PAULUS 2007). The female sex partners are imitated in shape and coloration of the labellum as well as in tactile stimuli such as surface structures, composition of epicuticular waxes, curvatures, firmness and pilosity patterns (BORG-KARLSON 1990). Trichomes on the lip surface were examined to give the impression of insect hairs and further cause the correct orientation of the pollinators on the labellum (PIRSTINGER 1996). All coloration patterns reflect UV light and were supposed to imitate insect wings (PAULUS 2007).

Fig. 3: Pseudocopulation: *Eucera longicornis,* male on *O. holoserica*, *Andrena nigroaenea,,* male on *O. sphegodes*, *Argogorytes mystaceus*, male on *O. insectifera* (PAULUS 2007)

1.6 First investigations on Ophrys

Although the flowers of *Ophrys* were always considered as being very exotic and strange, the biological meaning and reason behind that phenomenon was not investigated for a long time. Even the famous evolution-biologist Charles DARWIN, also known for his investigations concerning the pollination biology of orchids, only mentioned the genus *Ophrys* in a few lines in his book: “Fertilisation of orchids: The various contrivances by which orchids are fertilized by insects.” (1877). He cites some observations where several bees were supposed to be “attacking” the *Ophrys* flowers, treating them like some kind of “devil” that required to be “fought against”. Darwin claimed, that he could not explain that strange behaviour. Later, in the year 1916, the French A. Pouyanne first recognised the real reason behind this unusual phenomenon. He published an observation of a bee on a flower of *Ophrys speculum*, doubtless performing a copulation attempt on the labellum: “Un curieux cas de mimétisme chez les Ophrydées (*Ophrys*)” (POUYANNE 1917, PAULUS 2007).

2. Floral pigments

Among the angiosperms three major classes of floral pigments can be distinguished, anthocyanins, carotinoids and betalains (GROTEWOLD 2006, Fig. 4). Anthocyanins are mainly responsible for red, pink, purple, purplish and bluish flower colours, depending on the pH. The occurrence of the also red coloured betalains is restricted to the order of the Caryophyllales. in contrast to the lipophilic membrane-bound carotinoids, flavonoids and anthocyanins are both water soluble and thus located in the cell vacuoles (HARBORNE 1993, BUCHANAN et al. 2000).

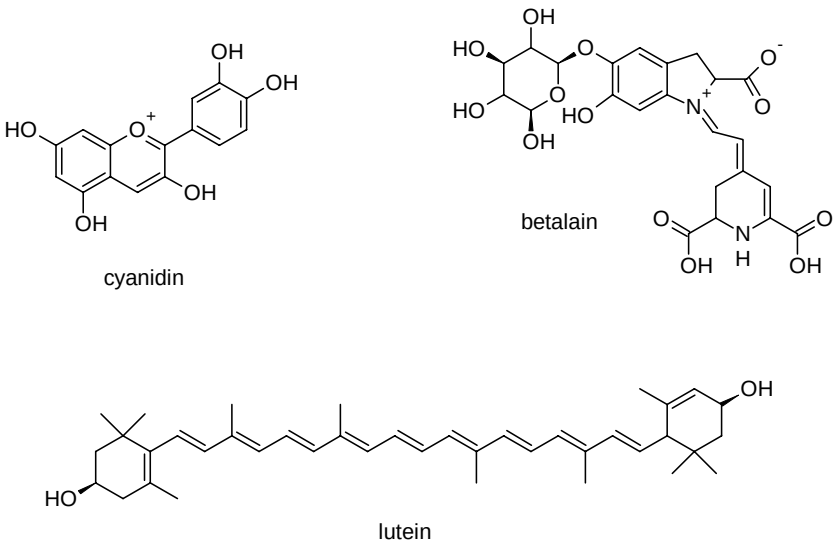

Fig. 4 The main floral pigments among the angiospermae, anthocyanidins (cyanidin), betalaines (betalain), and carotenoids (lutein)

2.1 Biosynthesis of flavonoids and anthocyanins

Anthocyanins are derivatives of the cinnamic acids. Cinnamic acid is derived from the amino acid phenylalanine, a product of the shikimi pathway, by phenylalanine ammonium lyase

(*PAL*).

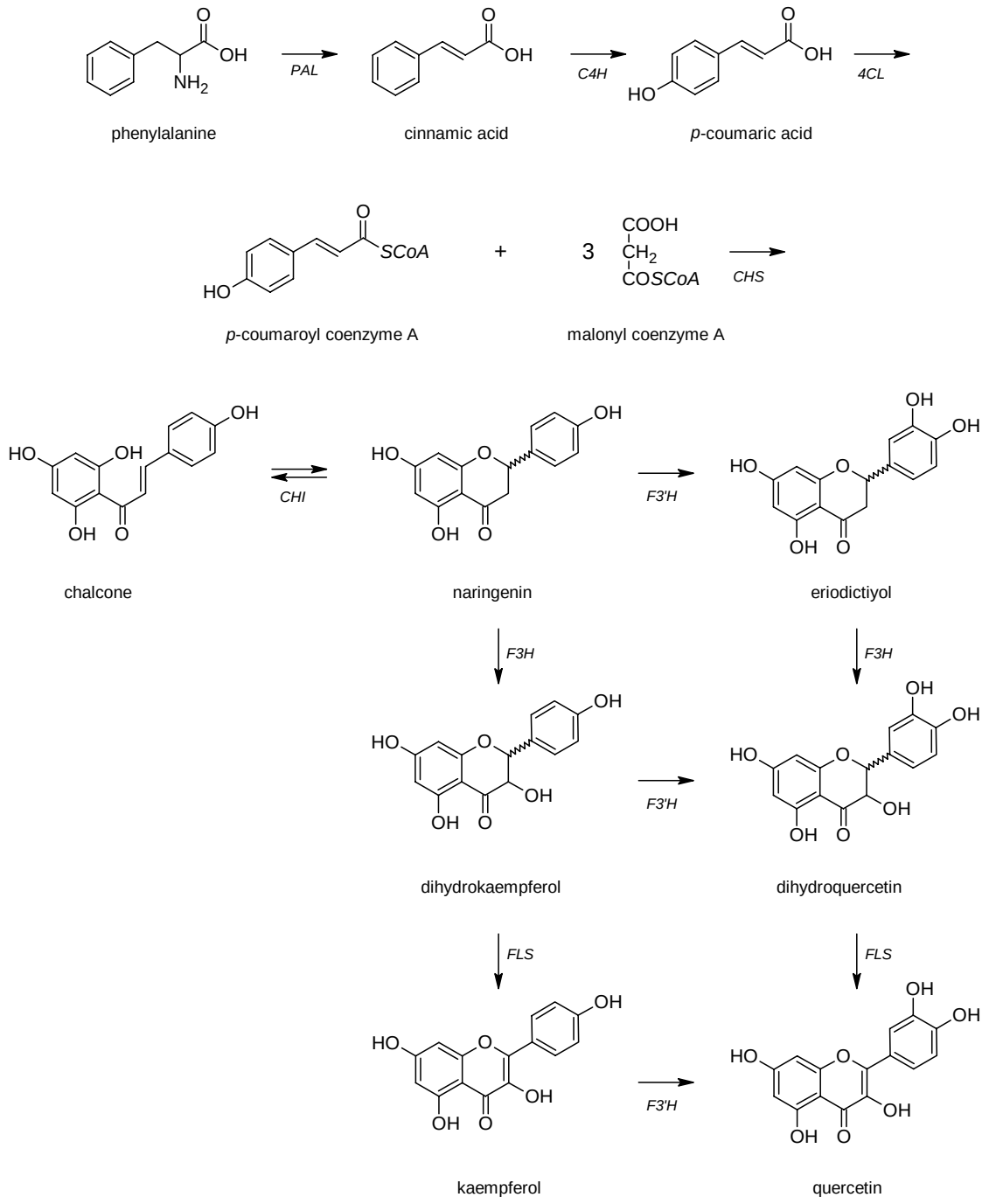

Fig. 5: Cinnamic acid biosynthesis, chalcone, flavanon and flavonol synthase. *PAL*, phenylalanine ammonium lyase; *C4H*, cinnamate 4-hydroxylase, *4CL*, 4-coumaroyl:CoA-ligase, *CHS*, chalcone synthase, *CHI*, chalcone isomerase, *FLS*, flavonol synthase, *F3H*, flavanone 3-hydroxylase;

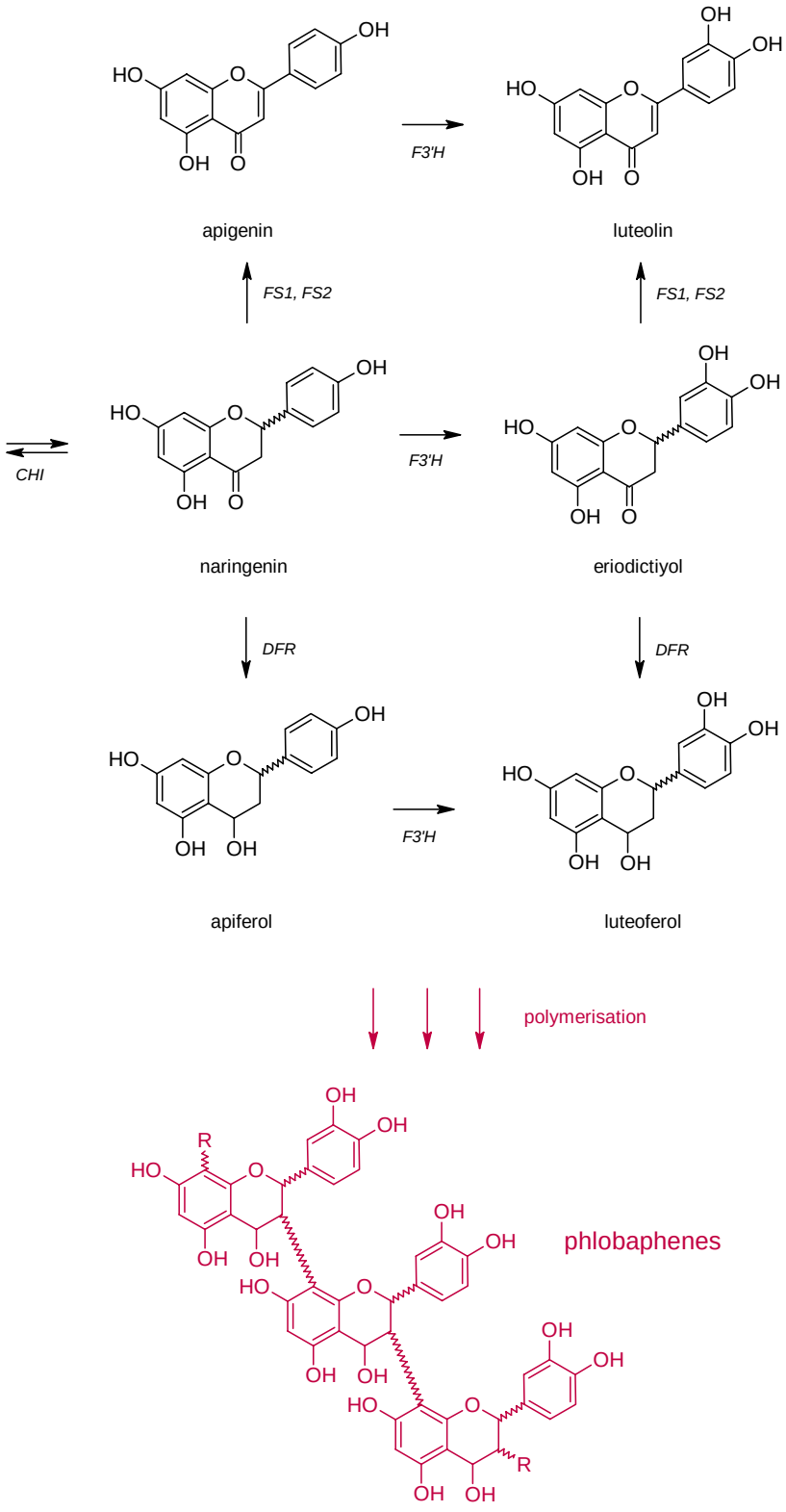

Fig. 6: Flavone biosynthesis and flavanones involved in phlobaphene polymerisation reaction. *F3´H*, flavonoids 3´-hydroxylase; *FS1*, *FS2*, flavone synthase 1, flavone synthase 2; *CHI*, chalcone isomerase ; *DFR* dihydroflavonol 4-reductase;

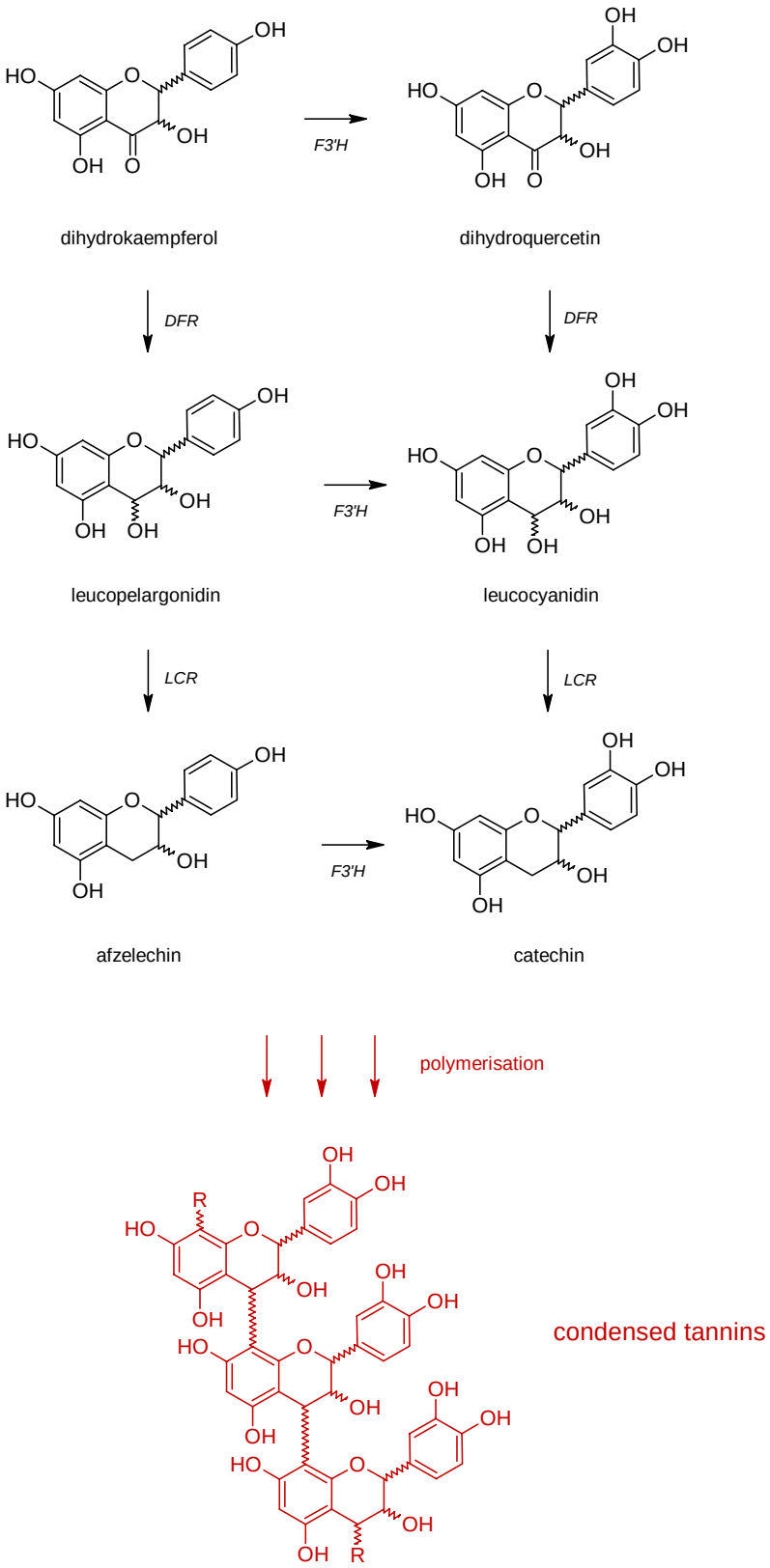

Fig.7: Catechin biosynthesis from dihydroflavonols and polymerisation reaction to condensed tannins. *F3´H*, flavonoids 3´-hydroxylase; *DFR*, dihydroflavonol 4-reductase; *LCR* leucoanthocyanin reductase;

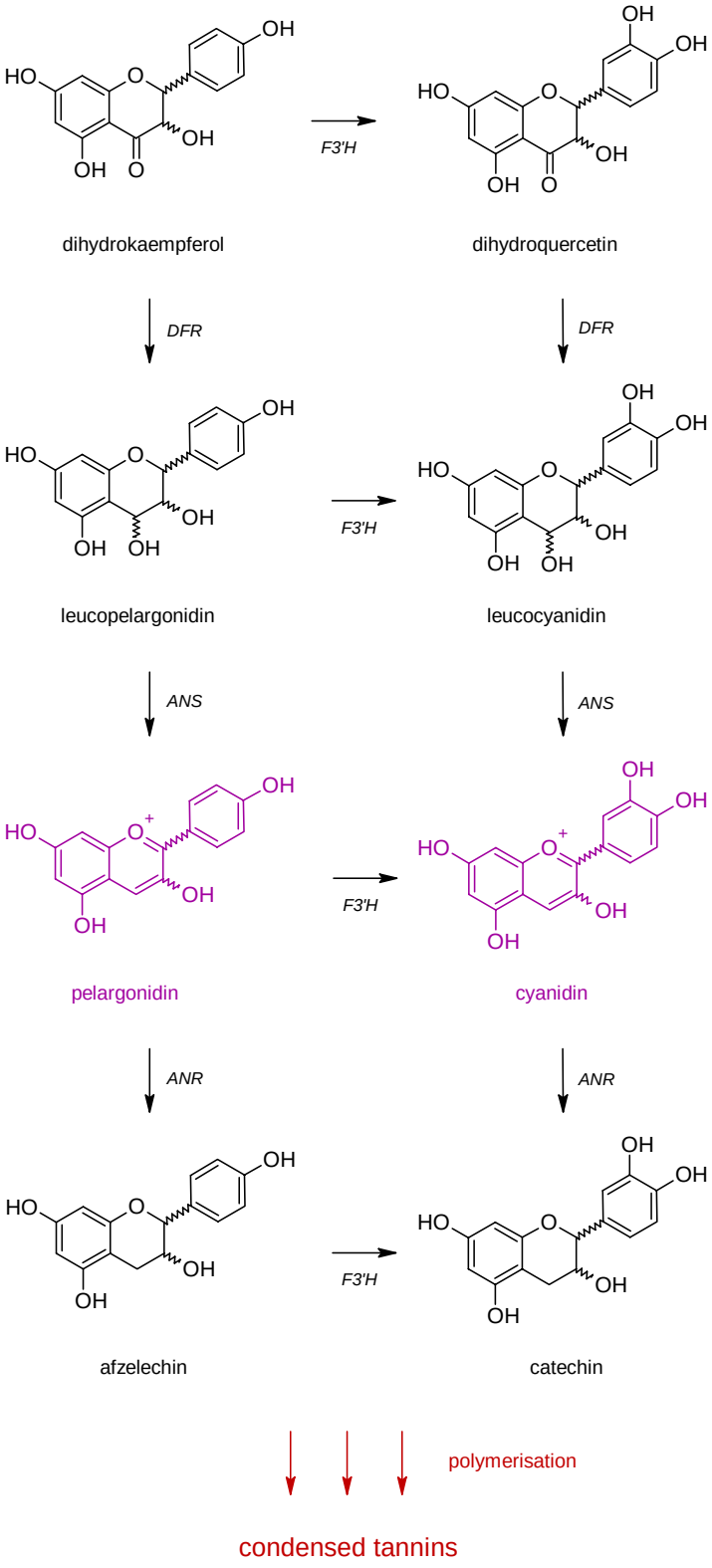

Fig. 8: Catechin biosynthesis via anthocyanins and polymerisation reaction to condensed tannins. *F3´H*, flavonoids 3´-hydroxylase; *DFR*, dihydroflavonol 4-reductase; *ANS*, anthocyanidin synthase, *ANR*, anthocyanidin reductase;

The enzyme phenylalanine ammonium lyase (*PAL*) deaminates phenylalanine by splitting off NH_4^+ ions. *Para*-coumaric acid, a 4-hydroxylated cinnamic acid derivative, serves as starter acid for the chalcone synthase in the flavonoid biosynthesis. Subsequently, three malonyl CoA units are attached to *p*-couma–royl CoA forming ring A of the tetrahydoxychalcone (TORSSELL 1983, KOES et al. 2005). Ring B of the pigment scaffold is contributed by the aromatic precursor acid (Fig. 5).

Flavanones are the precursors for the various types of different flavonoids, such as flavones, flavonols, isoflavones, and anthocyanins (TORSSELL 1997, WINKEL-SHIRLEY 2001). The enzymes flavone synthase 1 and 2 (FS1, FS2) catalyse the synthesis from flavanones to flavones. Furthermore, flavan-4-ols, e.g. apiferol or luteoferol, both subunits of the polymeric phlobaphenes, are also derived from flavanones by reduction catalyzed by dihydroflavonol -4 reductase (DFR) (WINKEL-SHIRLEY 2001) (see Fig. 6).

The catechins, subunits of the other important polymeric pigment class, the condensed tannins, are formed by reduction of either colourless leucoanthocyanins, e.g. leucopelargonidin or leudoanthocyanidin or the strong coloured anthocyanins. Leucoanthocyanidin reductase and anthocyanidin reductase are known as catalysts. Anthocyanins are synthesized via leucanthocyanins by the enzyme anthocyanidin synthase (WINKEL-SHIRLEY 2001, KOES et al. 2005, see Fig. 7and Fig. 8).

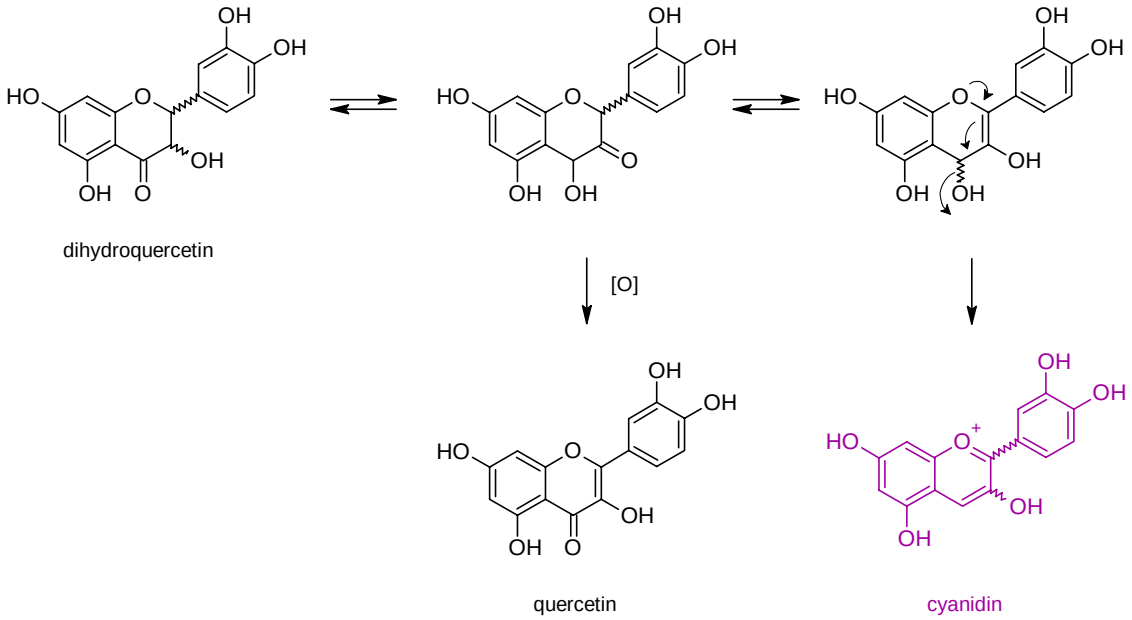

Fig. 9: Alternative possibility for anthocyanin and flavonol biosynthesis via dihydroflavonols.

An alternative anthocyanin pathway was published by TORSSELL, 1983 mentioning a synthesis of either flavonols, e.g. quercetin, or anthocyanins such as cyanidin via dihydroquercetin, a dihydroflavonol (Fig.9).

2.2 Chemical modification of flavonoids and anthocyanins

Sugars attached to the pigment scaffold increase the hydrophilic properties, whereas the plain conjugated ring structure, the aglycone, is lipophilic instead. Glycosyl moieties are most commonly linked to hydroxyl groups at C3, C5 or/and C7. Besides, O-methylation on the B-ring at C3', C4' or/and C5' may also occur (FORKMANN 1991, GROTEWOLD 2006). Vicinal hydroxyl and/or keto groups allow the formation of complexes with transition metal ions such as Al^{3+}, Fe^{3+}, Cu^{2+}. Acylation by addition of aromatic or/and aliphatic acids is recognised as

wide spread modification. Generally, cinnamic acid derivatives or malonic, oxalic or succinc acid are linked to the sugar units (REIN 2005). A further factors affecting pigment colour and stability is pH (HARBORNE 1993, BREINER 1999, GROTEWOLD 2006).

2.3 Flavonoids and anthocyanins in medicine

Both substance classes are known for their variety of positive effects on human health. Anthocyanins were already used by North American Indians, Chinese and Europeans in history as components of traditional herbal medicines (KONCZAK & ZHANG 2004). The polyphenolic structure is responsible for the scavenging of radical oxygen species (ROS) and nitrogen oxidative species (NOS). Free radicals are known to play an important role in the pathogenesis of many diseases, in particular cancer. Therefore, several scientific disciplines, such as medicine or physiology, devote increased attention to effects and properties of floral pigments (RICE-EVANS 2000).

2.4 Flavonoids and anthocyanins in orchid flowers

Anthocyanins are responsible for the pink and purple colour of the perigone of *Ophrys* flowers and all other orchids (ARDITTI & FISCH 1974). The pigmentation of *Ophrys* flowers in particular had been rarely examined. Nevertheless, anthocyanins were detected from a various number of other orchid species. Early investigations indicated a high diversity and a strong dependence on pH in regard to colour changes (BALL 1938). Recently, a study on acylated flavonoids revealed a huge chemical variety (TATSUZAWA et al. 1997, FIGUEIREDO et al. 1998).

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II. Function of floral pigments in the orchid genus *Ophrys*

Abstract

Individuals of *O. holoserica* and *O. untchjii* exhibit three different outer perigone colours, white, green and pink. *O. sphegodes* shows green outer tepals only. Accessions in two years from four sites were collected. Acidified methanol extracts of outer tepals were chromatographed over Amberlite XAD1180 and analyzed by HPLC/UV. Detected pigment compounds were classified by UV/VIS spectra and tentatively identified by an in-house spectra library and comparison to literature. HPLC profiles were analysed by principle component analysis (PCA) and compared to spectral reflection measurements. The redox activity of the extracts was determined by differential pulse voltammetry. Predominantly quercetin and kaempferol glycosides were detected in all samples throughout. Anthocyanins were exclusively found in traces in extracts of pink outer tepals. Nearly all samples displayed a presence of some quercetin and kaempferol glycosides, which were supposed to be acylated, as well as some cinnamic acid derivatives. Notable antioxidant activity was demonstrated in all samples throughout, although pink and green outer tepal extracts showed a higher redox activity than white. Spectral reflection appeared characteristic for each tepal colour and did not correlate with HPLC profiles. PCA showed, that the pigment composition was neither dependent on a certain tepal colour, nor on a particular site. A separate cluster was formed by pink outer tepals because of the presence of anthocyanins. Significant differences in pigment compositions did occur between accessions of different years, rather referring to a reaction to stress and a relation to different environmental conditions in the two years.

Keywords:

Flavonoids, anthocyanins, acylated flavonoids, cinnamic acids, antioxidant activity, spectral reflection

Introduction

Individuals of different species of the sexually deceptive orchid genus *Ophrys* show characteristic outer tepal colours. Two colour types can be distinguished: Some flowers exhibit a green outer perigone including *O. sphegodes*. By contrast, species ranked to the *O. holoserica – oestrifera* group present larger pink, pinkish or white outer tepals. (SPAETHE et al. 2007). However, in the aggregate of the latter species, this phenomenon was observed to occur between individuals belonging to one and the same species in some populations. For instance, different individuals of *O. holoserica* and *O. untchjii* are known to display white, green and pink tepal colours at the same site (DELFORGE 2005).

O. holoserica and the closely related *O. untchjii* are pollinated by the solitary bees *Eucera longicornis* and *E. clypeata, O. sphegodes* by *Andrena nigroaenea* (BAUMANN et al. 2006). Pollinators are supposed to be attracted to *Ophrys* flowers by visual and olfactory signals emitted from the labella. Tactile stimuli on the labella surfaces mimicking insect hairs guide the male visitors in a (pseudo-)copulation attempt (PRISTINGER 1996). During this action the body the pollinaria attach to the body of the insect and the pollen thus gets transferred if another flower is visited. The flowers do not provide nectar as reward (POUYANNE 1917, KULLENBERG 1961, PAULUS & GACK 2006, PAULUS 2008). Whereas the function of the

labellum in the sexual deception of the pollinators is clarified, potential contributions of the differently coloured tepals of the outer perigone have not been investigated yet.

The pigmentation of the outer tepals is caused by specific patterns of anthocyanins and flavonoids, both of which are derivatives of the flavonoid pathway (TORSSELL 1997). Methylation, glycosylidation and acylation create a huge structural diversity affecting light absorption and thus colour (HARBORNE 1993, MULDER-KRIEGER & VERPOORTE 1994, GROTEWOLD 2006). Caffeic, *p*-coumaric, ferulic, sinapic and chlorogenic acids but also hydroxybenzoic acid derivatives and aliphatic dicarboxyl acids such as malonic and oxalic acid comprise known acyl units (FIGUEIREDO et al. 1999, REIN 2005, VEITCH & GRAYER 2008). Further formation of oligomers further may occur between anthocyanins and flavonoids (CHEN & HRAZDINA 1981, BLOOR & FALSHAW 2000).

Polyphenols are also renowned scavengers of reactive oxygen (ROS), which are integral components of cellular signal cascades (HADDAD 2002). Vicinal diols on the ring B of flavonol and anthocyanin glycosides, e.g. quercetin and cyanidin, are responsible for the high antioxidant activity (PEITTA 1999, GOULD 2004). Free radicals are supposed to play an important role in the pathogenesis of many diseases. Not surprising, besides medicine and pharmacology, a wide range of research disciplines focuses on polyphenols (RICE-EVANS 2000, KONCZAK & ZHANG 2004).

In this study, extracts of all differently coloured outer tepals of *O. holoserica*, *O. untchjii* and green outer tepals of *O. sphegodes* were analysed by HPLC/UV to explore correlation of tepal colour with polyphenol composition. Accessions of individual flowers were obtained for two

consecutive years, 2007 and 2008, from 4 different sites. The profiling analyses aimed to assess if the composition of the polyphenols was determined by petal colour, species identity, or abiotic stress factors—the first year of the investigation period was characterized by an extensive drought period during flowering time. Furthermore, tepals of different colour were further investigated by reflections spectroscopy (attraction to pollinators) and differential differential pulse voltammetry (antioxidant potential). A strong correlation of petal colour with polyphenol patterns within a species and its populations was expected to provide evidence for a contribution of the outer tepals to the attraction of pollinators.

Material and methods

1. *Investigated Ophrys species*

The three species included in this investigation were as follows: *Ophrys holoserica* (Burm.f.) Greuter (Late spider orchid), *Ophrys untchjii* (Schulze M.) Delforge P. (Untchjii´s orchid) and *Ophrys sphegodes* Mill. (Early spider orchid) (Table 1).

2. *Extraction*

All three tepals of the outer perigone of each *Ophrys* flower were used for extraction and therefore carefully and completely removed by tweezers. The flower parts were placed in a clean mortar, covered with liquid nitrogen and grinded by hand using a pestle. The grinded plant material was then quickly submerged with methanol acidified with 1% acetic acid for a few minutes. Afterwards, each extract was transferred into a 2 mL Eppendorf cup, sonificated for 10 minutes and subsequently centrifuged (Biofuge Primo, Heraeus) for 5 minutes at 5000g min^{-1}.

3. *Sample preparation*

Each acidified methanol extract of 2 mL was diluted in 28 mL water. These suspensions were chromatographed over Amberlite XAD 1180 (Fluka, Buchs, Switzerland). Therefore, 2–3 g aqueous Amberlite-suspension were filled into glass columns of 1 cm diameter. The first

fraction was eluted using 50 mL of water, the second fraction was eluted with 70 mL of absolute ethanol, both acidified with 1% acetic acid. The ethanolic fractions were evaporated to dryness, weighed and dissolved in a mixture of methanol and water (1:1, v/v) acidified with 1% acetic acid.

4. HPLC analysis

The HPLC measurements were carried out using a Dionex Summit System (Dionex, USA) equipped with a photodiode array detector and a Famos autosampler (LC Packings, Netherlands). The column used was a Phenomenex Synergi Max C18 (150 x 2 mm). The particle size of the stationary phase was 5 µm. The column oven was adjusted to 40°C and the flow rate was adjusted to 0.2 mL min^{-1}. Solvent A was prepared as follows: Water (Milli Q quality) /methanol /*o*-phosphoric acid (H_2O : CH_3OH : H_3PO_4 = 9:1:0.5, v/v/v), solvent B was pure methanol. The gradient was started with 100% of solvent A for 2 minutes and subsequently changed linearly to 100% of solvent B within 98 minutes. Finally, this concentration was held for further 10 minutes (Table 2). Five µL of each sample were injected. The UV-spectra were recorded from 220nm to 590nm. Tentative assignment of structures was carried out on basis of comparison with an in-house UV spectra library and further comparison with data from literature.

5. *Differential Pulse Voltammetry*

Extracts of the outer perigone of *O. holoserica* and *O. untchjii* were used for measurements. Voltammetric analyses included differential pulse voltammetry (DPV) as well as cyclic voltammetry (CV). The thus obtained voltammograms aimed at comparing the redox activities of the various pigment extracts of differently coloured outer tepals (white, green, pink) of the two *Ophrys* species.

The dried extract was dissolved in 10 mL 0.2 M acetic acid buffer (pH = 3.6). The low pH was chosen in order to protect the pigment compounds against oxidative decomposition and polymerization. The phenolic compounds are originally located in the cell vacuoles of the tepals in an acid milieu. For all samples, the concentration was 0.5 mg/mL. All solutions were degassed by argon for at least 10 minutes prior to the electrochemical experiments. DPV was measured at room temperature using a three-electrode system µAutolab type III potentiostart/galvanostat PGSTAT (EcoChemie Inc., The Netherlands). The electrode-system consisted of a glassy carbon working electrode of 3 mm diameter, an Ag/AgCl (saturated KCl) reference electrode and finally a platinum wire as a counter electrode. The glassy carbon working electrode was polished before each measurement. The differential pulse voltammetry was carried out with the adjustment of following parameters throughout: An initial potential of –300 mV, an end potential of +1300 mV, a modulation amplitude of 25 mV, a step potential of 5 mV and a interval time of 250 ms. The antioxidant activity was calculated as the sum of the oxidation peaks and their respective area. Comparison of the voltammograms was carried out by standardization of the extract amounts to a concentration of 5 mg.

6. Reflection spectroscopy

Emission spectra of tepals were measured for all three *Ophrys* species, *O. holoserica*, *O. untchjii* and *O. sphegodes*. The spectral reflection (emission) of the different coloured tepals of the outer perigone (white, green or pink outer tepals) was measured using a USB 2000 spectrometer (Ocean Optics B.V., Netherlands). Calibration was performed before each measurement using a PTFE white standard (CHITTKA and KEVAN, 2005). Small, ca. 0.25cm^2 tissue pieces (ca. 1 or 2 tepals respectively) were sufficient for analysis. For visualization, SigmaPlot 10.0. (Sysdat Inc., USA) was used.

7. Statistics

A chemometric analysis of the HPLC-detection signals (229 nm) of both sample collections (2007 and 2008) was carried out by principal component analysis (PCA). All statistical analyses were carried out using SIMCA_P 11 (Umea, Sweden).

Results

1. HPLC analyses of outer tepal pigment compounds

All outer tepal extracts of the accessions collected in 2007 and 2008 were analyzed by HPLC and yielded quite similar chromatogram profiles (Fig. 1). Significant peaks eluted between around 12.00 min and 50.00 min of the solvent gradient. The investigated flower pigment compounds were predominantly glycosides due to their elution with eluent mixtures of water content of more than 50%. The HPLC showed no notable peaks of lipophilic pigments such as carotenoids.

Apart from anthocyanins, the pigment composition of the white, green and pink outer tepal extracts was quite similar to each other. Significant differences between the three different perigone types were not detected. However, analyses of sample collections from both years formed separate clusters in the PCA analysis. HPLC analyses of the extracts of the 2008 accessions afforded more detected peaks in comparison to the analyses carried out in 2007.

1.1 Flavonoid glycosides

Flavonoids turned out as the most dominant among the detected peaks. They eluted between 21.00 and 40.00 min retention-time. On basis of UV-spectra, two structural types were recognised: Several peaks were detected as quercetin, other peaks as kaempferol glycosides. The UV spectra of the two flavonol derivatives differed in shape and position of maxima.

Kaempferol and quercetin derivatives were found in all analyzed samples. The large number of peaks can be explained by variable sugar moieties linked to ring A and ring C of the flavonoid scaffold. Eight quercetin- and six kaempferol derivatives were detected (Fig. 2, Fig. 3).

1.2 Anthocyanins

In contrast to flavonoids, anthocyanins occurred only in traces despite their decisive effect on tepal colour. Due to low concentrations, only a small number of those pigment compounds was actually detectable. Most of them appeared in the flanks or shoulders of peaks of co-eluting flavonoids between 24.00 and 31.00 min retention-time. The six recognised anthocyanins were only detectable by careful examination of all peak spectra. An exact identification was not possible. The most notable characteristic of the UV spectra of anthocyanins is a broad absorption maximum at higher wavelengths between 514 and 540 nm (Fig. 4). Anthocyanidins were exclusively detected in extracts of *Ophrys holoserica* and *Ophrys untchjii* outer tepals with clearly pink colour.

1.3 Cinnamic acid derivatives

On basis of the UV library spectra, several cinnamic acid derivatives could be identified between 14.00 and 23.00 min, though, compared to the flavonoids, in minor amounts. The latest eluting derivative was chlorogenic acid. Interestingly, *o*- and *m*-coumaric acid chromophores eluted as a pair of peaks (Fig. 5).

1.4 Acylated flavonoid glycosides

In addition to the mentioned flavonol glycosides, other structures were detected which obviously related to flavonoids. The presence of cinnamic acid derivatives reflected itself in the additional maxima in the UV spectrum of those flavonoid-like structures. This strongly suggested the presence of acylated flavonoid glycosides, which were found in all extracts independent of tepal colour and species (Fig. 6).

1.5 Non-identified peaks

All HPLC chromatograms exhibited a remarkable high peak around 35.00 min retention time, which somehow resembled benzoic acid, a simple aromatic compound. However, the retention time differed by 5 min. This peak and many others, which were not unambiguously assigned as flavonoids, were not included into the statistical analysis.

2. Principle component analysis of detected phenolic compounds

The peak patterns obtained from the HPLC analyses of all accessions were analyzed by a principal component analysis to explore the correlations peak patterns. No clusters were obtained for outer tepal colour (Fig. 10, A) or accession site (Fig. 10, B) However, the accessions of 2007 and 2008 did form two distinct clusters (Fig. 10, C). The differences between the two years were caused by different accumulation patterns of specific cinnamic acids, kaempferol and quercetin flavonoid glycosides and acylated glycosides. Pronounced accumulation of anthocyanins occurred only in the 2008 accessions. However, the clear

clustering of the accessions of each year (first component in Fig. 11) was caused by pattern differences within all focussed types of phenols. The presence of anthocyanins in the accessions of pink coloured tepals in 2008 led to a separate clustering of these accessions. In 2007, anthocyanins were only detected in some accessions of the pink coloured tepals, and if, only in traces. Consequently, no distinct clustering was obtained for the pink outer tepals in 2007.

2.1 Ophrys sphegodes

The green outer tepal extracts of *O. sphegodes* did not contain any anthocyanins. The patterns of the quercetin and kaempferol glycosides as well their acylated derivatives were rather variable (Fig. 12) and clustered with the accessions of the white and pink coloured accessions of *O. holoserica* from the same year (Fig. 10a).

2.2 Ophrys holoserica

In 2007, accessions from two localities, St. Georgen and Perchtoldsdorfer Heide, were analyzed (Fig. 13 and 14). The variability in the phenol patterns precluded separate clustering (Fig. 10a–c) neither on basis of the accession site or on tepal colour. In 2008, white, green and pink coloured outer tepals were analyzed. The pink outer tepals accessions clearly differed by more pronounced anthocyanin accumulation than in the previous year correlating with the more notable pink pigmentation (Fig. 15). Interestingly, also variants with green outer tepals were found, which showed a tendency to cluster separately from the white accessions. An analysis of the score contributions of the PCA indicated that, in comparison to the average, a

tendency to accumulate cinnamic acid derivatives was higher than in white coloured petals (Fig. 16).

2.3 Ophrys untchjii

In 2008, accessions from *O. untchjii*, which were available. The pink accession exclusively accumulated anthocyanins (Fig. 17). Two of the three green accessions followed the trend to accumulate cinnamic acids already observed for the Austrian green tepal individuals of *O. holoserica* (Fig. 16). Notably, accessions from both the Austrian and Croatian locality clustered together despite the pronounced geographic distance (Fig. 10a – 10c).

3. Differential pulse voltammetry (DPV) of outer tepal extracts

The investigated *Ophrys* species, *O. holoserica* and *O. untchjii*, demonstrated antioxidant activity in their outer tepal extracts throughout. All measured samples yielded quite similar DPV voltammograms indicating three peaks at around 0.4 V, 0.9 V and 1.1 V. Indeed, variances between the three different outer tepal colours were recognised. Pink(ish) outer perigone extracts showed the highest peaks at 0.4 V. Instead, in extracts of white tepals this one was the lowest. Concerning the intensity of this peak, the resulting voltammograms of the green outer tepal extracts were in between the other two coloured samples. In general, all measured white tepal extracts showed the lowest antioxidant potential in comparison to the differently coloured samples. The mentioned peaks at 0.9 V and 1.1 V representing a further oxidation of extract components appeared to be less prominent on the voltammograms

compared to the peak at 0.4 V. However, these two peaks were found in all measured samples throughout (Fig. 9).

4. Reflection spectroscopy

All flowers possessing the same outer tepal colour yielded similar reflection spectra, independent of a certain species or a certain accession site. *Ophrys* flowers exhibiting white outer tepals did not cause any specific strong reflection maxima. Actually, the whole visible light range was reflected entirely thus leading to an almost horizontal spectral reflection curve between 440 nm and 700 nm. (Fig. 19 A, Fig. 22 A) Flowers with green and pink outer perigones afforded colour-specific reflection maxima. Pink outer tepals demonstrated two strong spectral reflection maxima at around 450 nm in the blue light range as well as in the orange/red light range at around 620 nm. Wavelengths around 550 nm were absorbed instead (Fig. 19 B, Fig. 22 C). By contrast, green outer perigones only caused a single but also strong reflection maximum at 550 nm right in the green visible light range, whereas wavelengths around 450 nm and 620 nm were absorbed (Fig. 20, Fig. 22 B, Fig. 23). Pink and green outer tepals displayed opposite reflection and absorption to each other, white outer tepals nearly reflect all colour light ranges (Fig. 21, Fig. 24).

Discussion

Flavonol derivatives, quercetin and kaempferol glycosides, were the most notably detetable phenols in the outer tepals of the various investigated *Ophrys* species, as proven by their characteristic UV spectra. In previous studies, quercetin and kaempferol glycosides were also detected in tepals of *Dendrobium sp*. and various other orchids (HARBORNE & WILLIAMS 1998, WILLIAMS et al. 2001). In a recent study, the occurrence of kaempferol glycosides was confirmed in the outer tepals of some *Ophrys* species not included within this study by comparison with reference compounds. Epiflourescence microscopy observations indicated a strong accumulation of flavonoids in the protoplasts and cell walls of the tepal epidermis cells as well as in the subcuticular cell layers (KARIOTI et al. 2008). Acylated flavonoids have not been analysed in tepals of *Ophrys* so far, but they are well known to occur in flower organs; cinnamic acid derivatives, *p*-, or *m*-coumaric acid, are supposed to be linked to quercetin and kaempferol glycosides (VEITCH & GRAYER 2008, CUNNINGHAM & EDWARDS 2008). Anthocyanins, though occurring in rather low concentrations compared to the flavonol glycosides, were responsible for causing a sometimes rather intensive pink colorization of outer tepals of some individuals of *O. holoserica* and *O. untchjii*. Previous studies report chrysanthemin (cyanidin-3-monoglycoside) to occur in outer tepals of *O. insectifera* and *O. apifera* (UPHOFF 1979). Ophrysanin (cyanidin-3-oxalylglycoside) was shown to be characteristic for the genus *Ophrys* and was suggested to constitute the precursor for more complex acylated cyanidin glycosides (STRACK et al. 1989). A related structure named orchicyanin I (cyaniding oxalyl-3,5-diglycoside-kaempferol-7-glycoside) was elucidated from tepal extracts of *O. holoserica* and several other *Ophrys* species by the same authors. *Para*- and *o*-coumaric acid were detected in almost every extract analyzed. Chlorogenic acid was

frequently found in the analyzed samples as well. This aromatic acid is an ester between caffeic acid and quinic acid and may also occur as acyl moiety of flavonol glycosides (DAVIS & MAZZA 1993).

Specific patterns of the investigated flavonoid glycosides neither correlated with a specific outer perigone colour nor a specific species or particular accession site. Anthocyanins, which exclusively occurred in traces in the extracts of tepals of *O. holoserica* and *O. untchjii* exhibiting a pronouncedly pink outer perigone colour caused specific minima in the reflection spectra. The green coloured tepals also showed characteristic reflection minima, which were caused by higher amounts of chloroplast pigments, such as chlorophyll and carotenoids, likewise irrespective of the investigated species and accession site.

A principal component analyses of the of the relatively quantified peak areas if the HPLC-detected phenols revealed only a pronounced clustering of the accessions according to the year of collection. This may be interpreted as a consequence of different environmental conditions. An extensive drought period in April 2007 caused disadvantageous growing conditions and earlier flowering time for orchids in general. In 2008, regular rainfall and lower average temperatures in spring favoured the development *Ophrys* inflorescences. The higher number of flowering individuals revealed a higher variation in petal colour, even green and dark pink variants were detected at the accession site Perchtoldsdorfer Heide.

Differential pulse voltammograms of the outer tepal extracts all displayed similar oxidation peaks, which were most probably caused by the kaempferol and quercetin flavonol glycosides. For the latter, antioxidant activity was particularly reported (GUOHUA et al. 1996, LEOPOLDINI

et al. 2006). The hydroxyl groups on ring B of quercetin and the cyanidin derivatives supposedly contribute to the peaks at 0.4 V on the voltammograms. Pink outer tepal extracts showed the highest peaks at this position, reflecting the presence of cyanidin derivatives. Further peaks at 0.9 V and 1.1 V may mark an oxidation of ring A or C of the flavanols. The slightly more pronounced oxidation peaks of the green outer tepal extracts may be caused by a trend to higher accumulation of cinnamic acid derivatives as pointed out by the PCA analysis.

A recent study on pollinator behaviour towards different coloured outer tepals of *Ophrys* indicated a preference of flowers exhibiting pink coloured outer tepals. However, the authors interpreted this as a secondary attraction phenomenon and attributed more importance to floral odour (SPAETHE et al. 2007). The scent emits from the cuticle of the labellum and matches the female sex pheromone of the pollinating species. The olfactory cues were analyzed by GC/MS in several studies (BORG-KARLSON 1990, SCHIESTL et al. 1999, 2000, AYASSE et. al. 2003). Visual signals from the labellum alone did not attract any pollinators at all (PAULUS 2007). The results obtained in this study concur with these insights and rather suggest that the pronounced accumulation of phenolic compounds in the tepals may have evolved contributing to the tolerance of stress caused by high light intensities or draught in the first place. Accordingly, the pronounced clustering of the accessions obtained in the two years, irrespective of tepal colour, species or accession site, represents the most convincing evidence.

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Tables & Figures

Ophrys species	population	country	date (DD.MM.YY)	tepal colour	Extract dry weight [mg]
Sample collection 2007					
O. *holoserica* 1	Perchtoldsdorfer Heide (Vienna)	A	10.05.07	white	n.d.
O. *holoserica* 2	Perchtoldsdorfer Heide (Vienna)	A	10.05.07	white	n.d.
O. *holoserica* 3	Perchtoldsdorfer Heide (Vienna)	A	10.05.07	white	n.d.
O. *holoserica* 4	Perchtoldsdorfer Heide (Vienna)	A	10.05.07	white	n.d.
O. *sphegodes* 5	Bisamberg (NÖ)	A	11.05.07	green	n.d.
O. *sphegodes* 6	Bisamberg (NÖ)	A	11.05.07	green	n.d.
O. *sphegodes* 7	Bisamberg (NÖ)	A	11.05.07	green	n.d.
O. *sphegodes* 8	Bisamberg (NÖ)	A	11.05.07	green	n.d.
O. *sphegodes* 9	Bisamberg (NÖ)	A	11.05.07	green	n.d.
O. *holoserica* 10	St. Georgen (BGLD)	A	18.05.07	white	n.d.
O. *holoserica* 11	St. Georgen (BGLD)	A	18.05.07	white	n.d.
O. *holoserica* 12	St. Georgen (BGLD)	A	18.05.07	white	n.d.
O. *holoserica* 13	St. Georgen (BGLD)	A	18.05.07	white	n.d.
O. *holoserica* 14	Perchtoldsdorfer Heide (Vienna)	A	18.05.07	pink	n.d.
O. *holoserica* 15	Perchtoldsdorfer Heide (Vienna)	A	18.05.07	pink	n.d.
O. *holoserica* 16	Perchtoldsdorfer Heide (Vienna)	A	18.05.07	pink	n.d.
Sample collection 2008					
O. *untchjii* 1	Premantura, South Istria	HR	13.05.08	white	n.d.
O. *untchjii* 2	Premantura, South Istria	HR	13.05.08	green	n.d.
O. *untchjii* 3	Premantura, South Istria	HR	13.05.08	pink	n.d.
O. *holoserica* 4	Perchtoldsdorfer Heide (Vienna)	A	20.05.08	white	4.2
O. *holoserica* 5	Perchtoldsdorfer Heide (Vienna)	A	22.05.08	green	2.1
O. *holoserica* 6	Perchtoldsdorfer Heide (Vienna)	A	20.05.08	pink	1.7
O. *holoserica* 7	Perchtoldsdorfer Heide (Vienna)	A	20.05.08	white	6.2
O. *holoserica* 8	Perchtoldsdorfer Heide (Vienna)	A	20.05.08	white	7.0
O. *holoserica* 9	Perchtoldsdorfer Heide (Vienna)	A	20.05.08	white	11.1
O. *holoserica* 10	Perchtoldsdorfer Heide (Vienna)	A	20.05.08	white	n.d.
O. *untchjii* 11	Premantura, South Istria	HR	13.05.08	green	n.d.
O. *untchjii* 12	Premantura, South Istria	HR	13.05.08	green	5.9
O. *holoserica* 13	Perchtoldsdorfer Heide (Vienna)	A	22.05.08	green	5.1
O. *holoserica* 14	Perchtoldsdorfer Heide (Vienna)	A	22.05.08	green	6.3
O. *holoserica* 15	Perchtoldsdorfer Heide (Vienna)	A	20.05.08	pink	4.0
O. *holoserica* 16	Perchtoldsdorfer Heide (Vienna)	A	20.05.08	pink	3.8
O. *holoserica* 17	Perchtoldsdorfer Heide (Vienna)	A	20.05.08	pink	4.5
O. *holoserica* 18	Perchtoldsdorfer Heide (Vienna)	A	20.05.08	pink	n.d.

Table 1: Acessions of Ophrys species and dry weigth of extracts (n.d. = not determined)

time [min]	solvent A: water/methanol/*o*-phosphoric acid (9:1:0.5; v/v/v) [%]	solvent B: methanol [%]
0	100	0
2	100	0
98	0	100
120	0	100

Table 2: HPLC measurements: solvent gradient.

HPLC – analysis

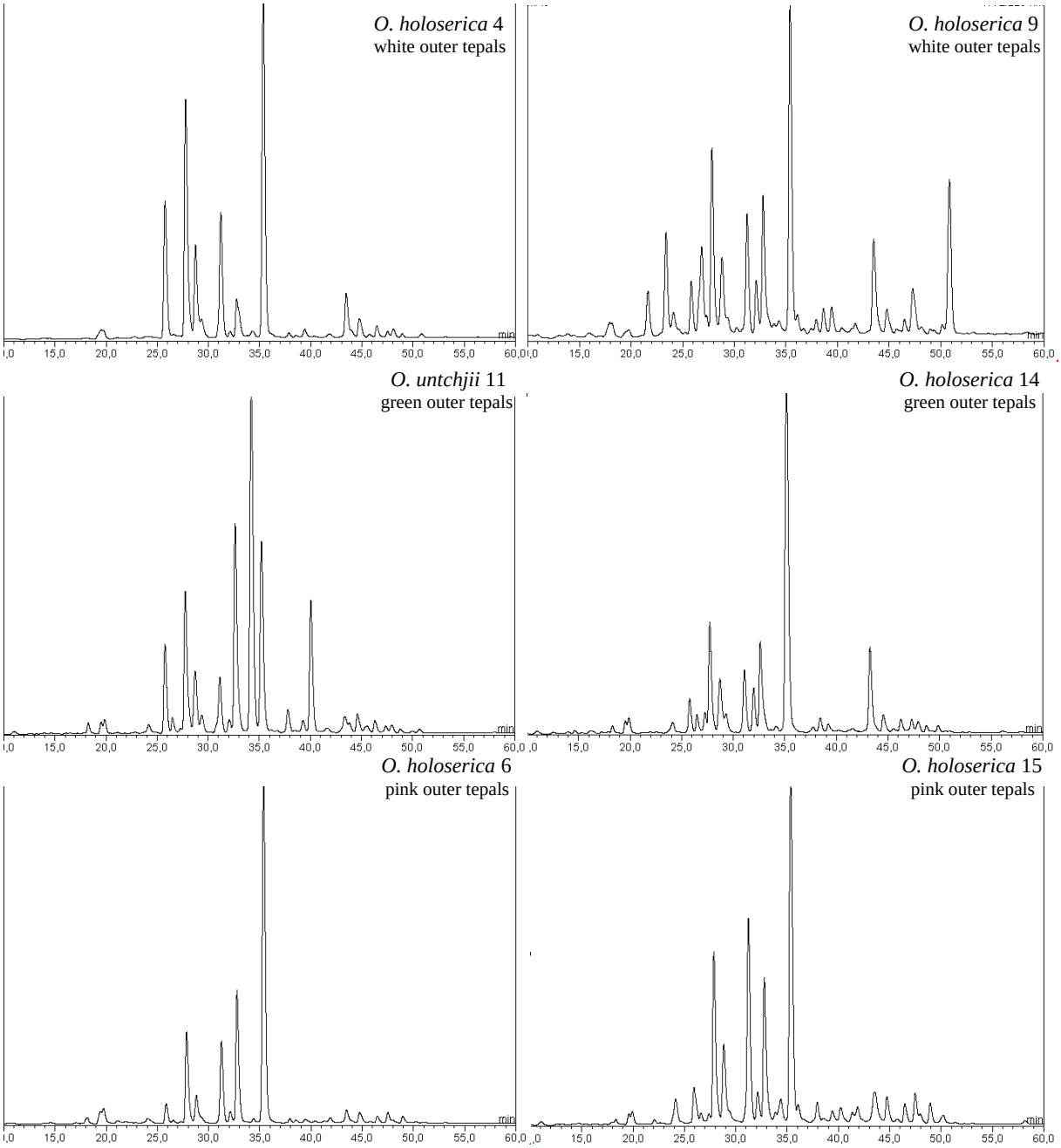


Fig. 1: HPLC of methanol/water outer tepal extracts (1/1; v/v), Accessions 2008.

Flavonoids: Quercetin glycosides

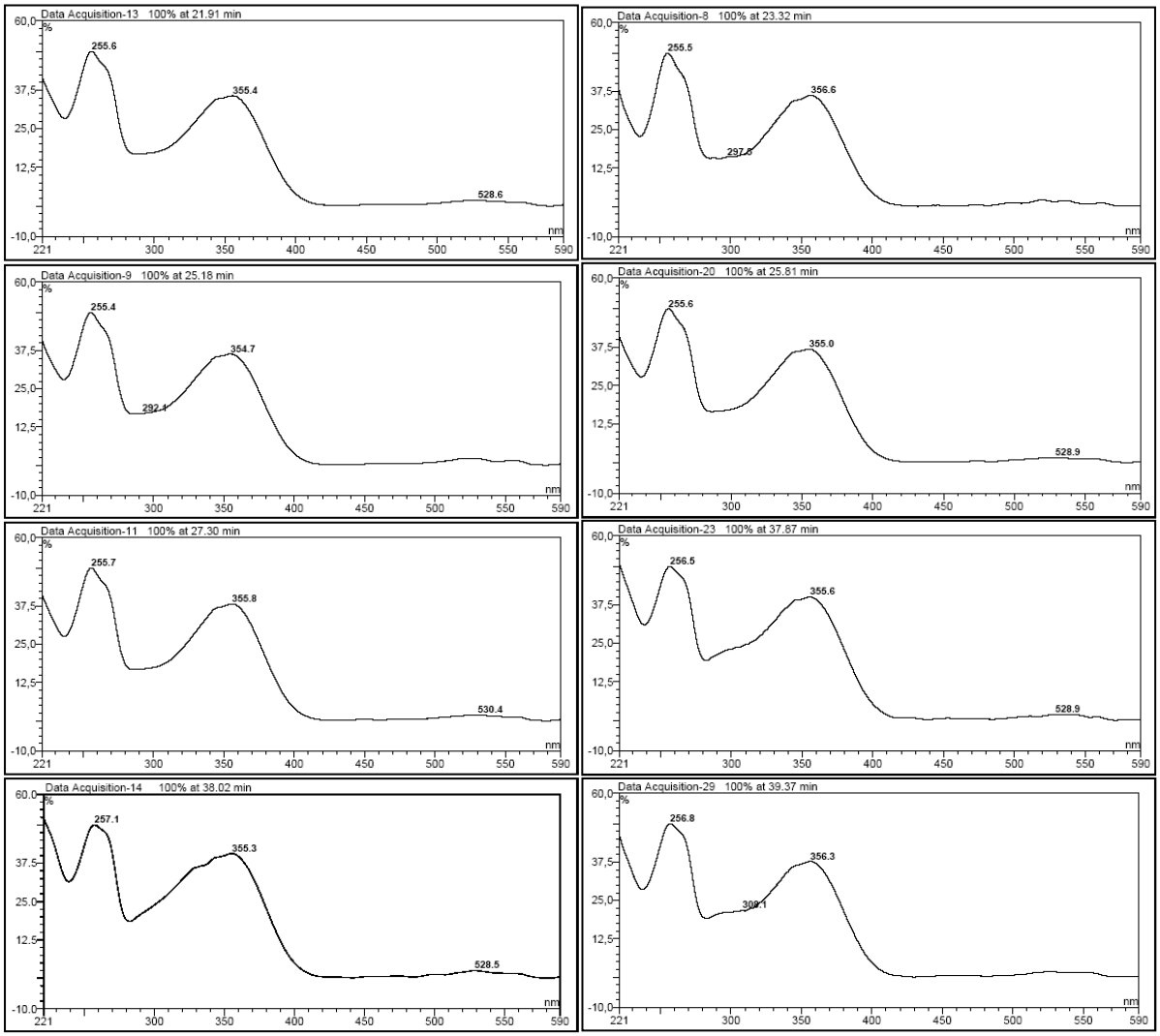


Fig. 2: UV spectra of quercetin glycosides

Flavonoids: Kaempferol glycosides

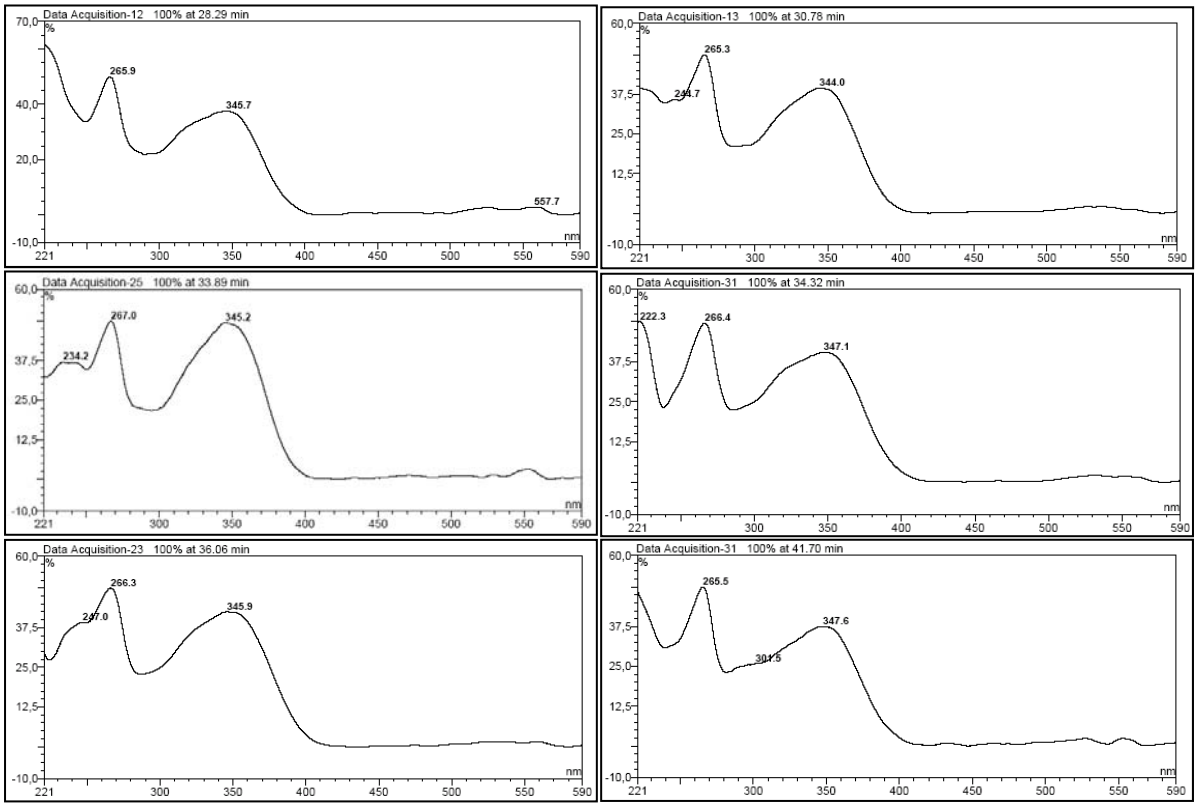


Fig. 3: UV spectra of kaempferol glycosides

Anthocyanins

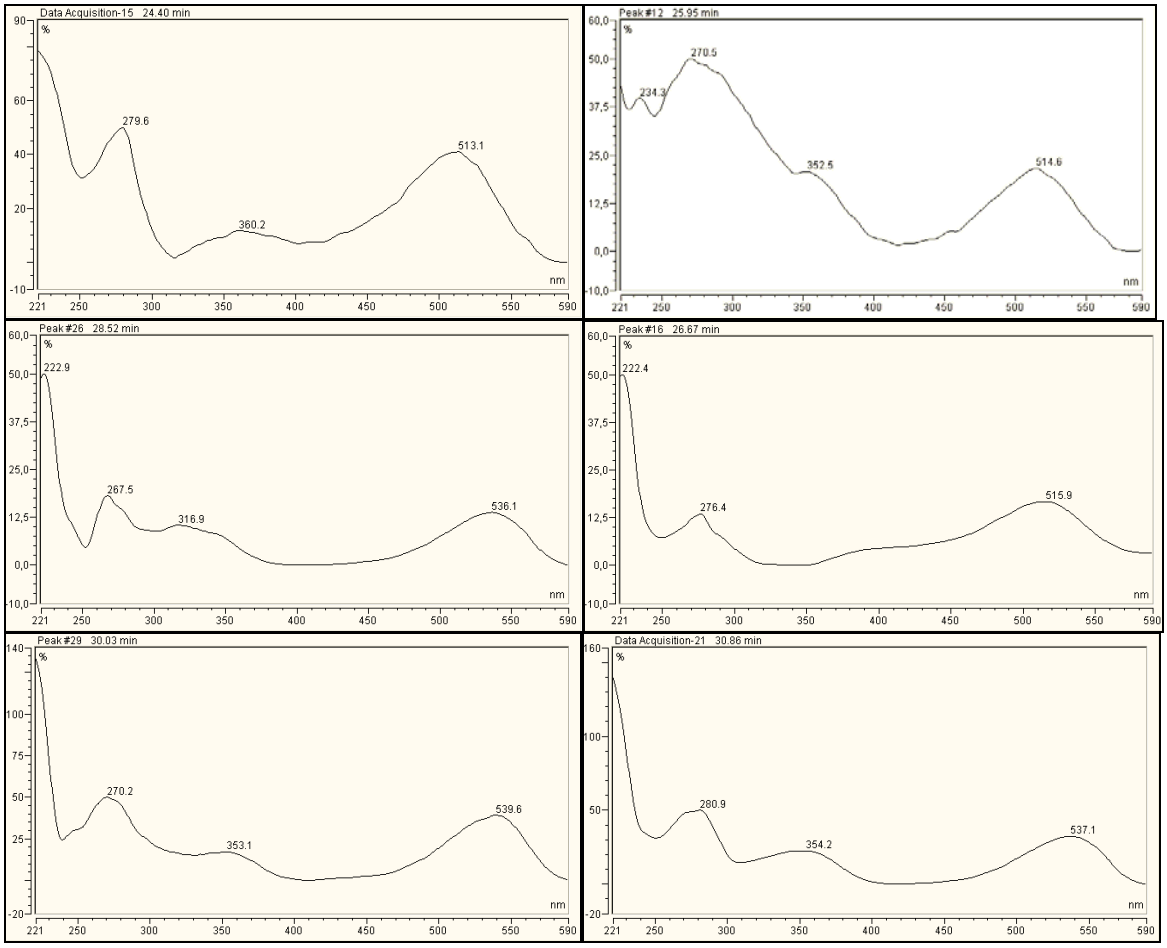


Fig 4: UV spectra of anthocyanins

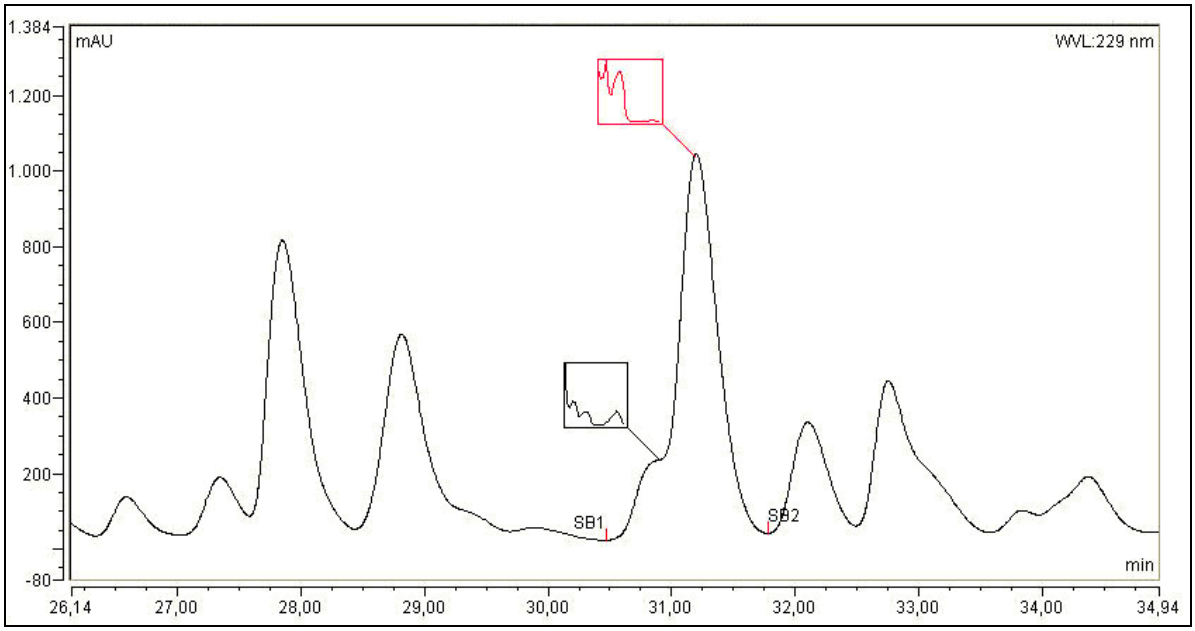


Fig 5: Anthocyanins were detected in the peak shoulders of kaempferol glycosides

Phenylpropanoids: Cinnamic acids

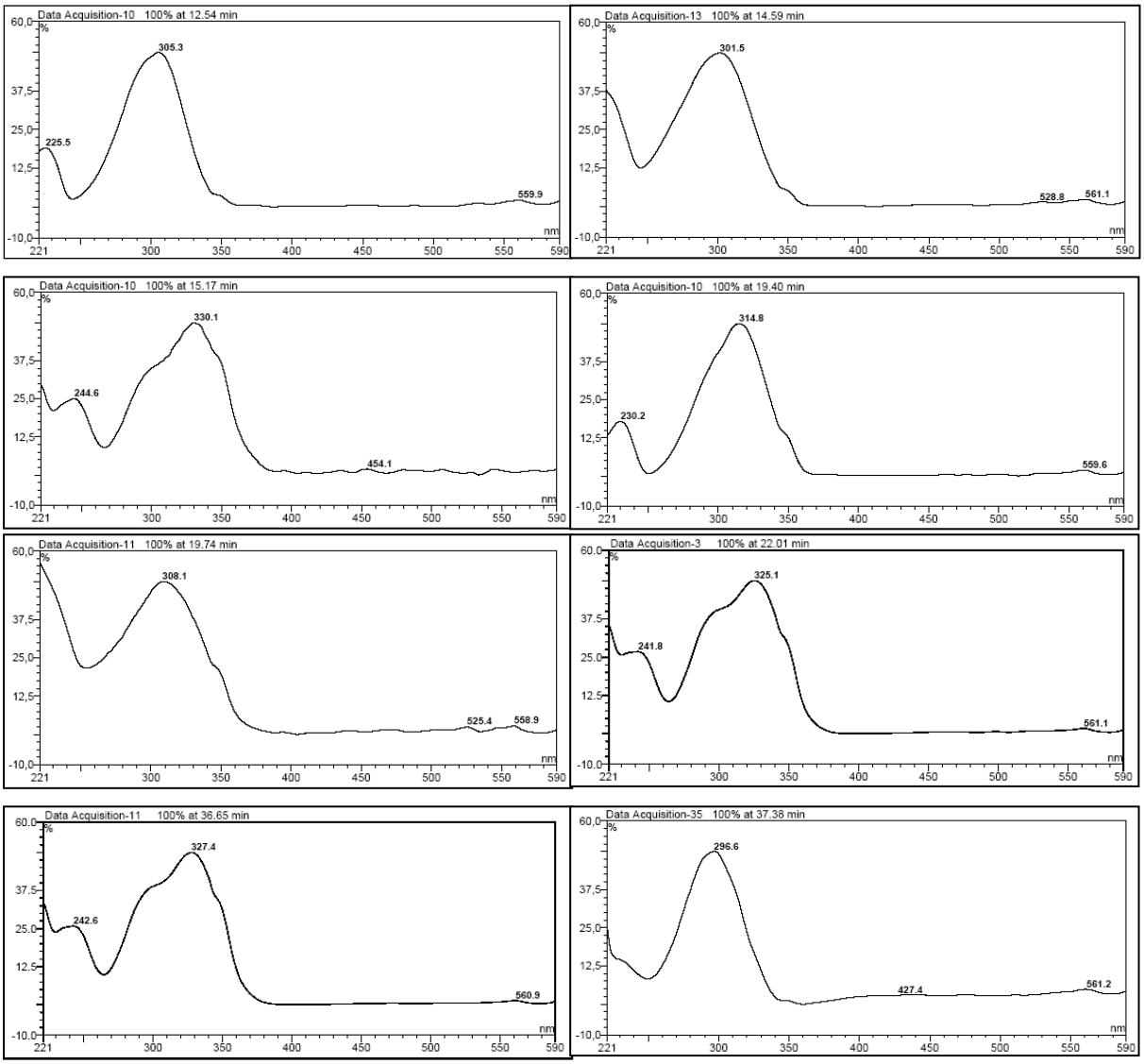


Fig. 6: UV spectra: phenolic acids

Acylated flavonoids

1) Quercetin glycosides /cinnamic acids:

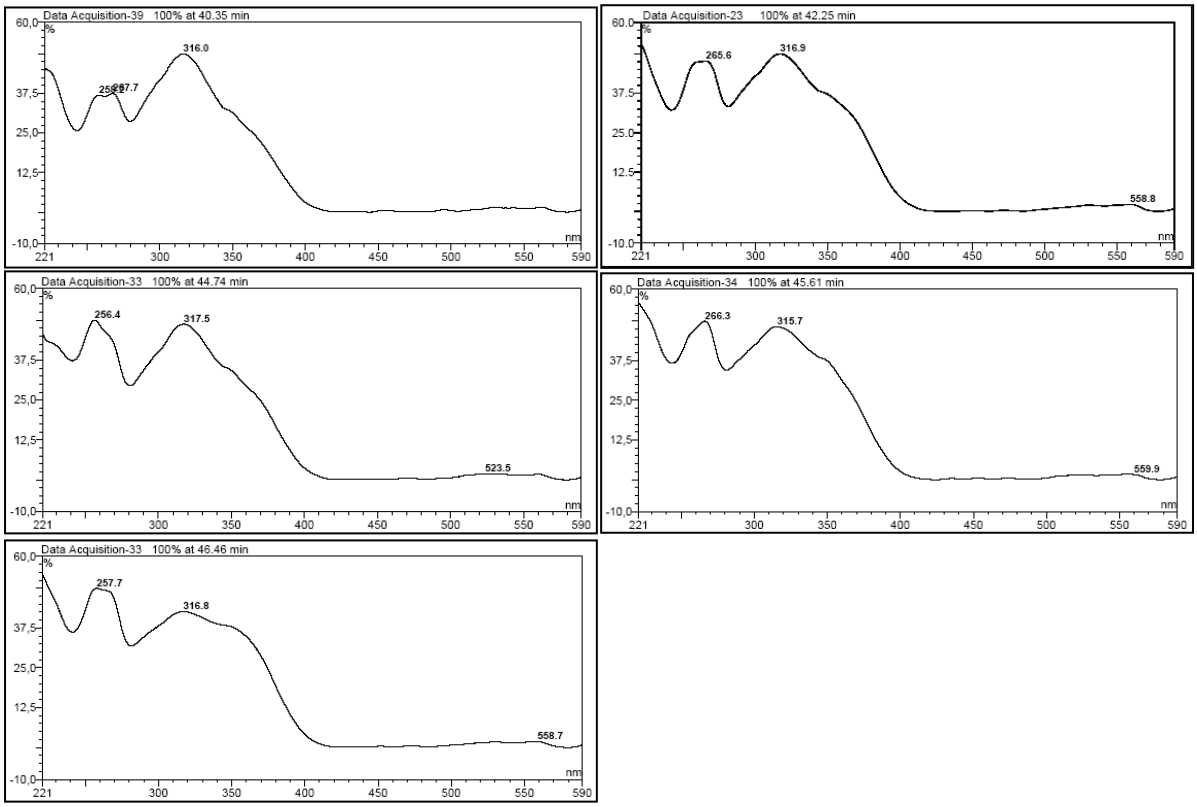


Fig. 7: UV spectra: Acylated quercetin derivatives

2) Kaempferol glycosides/cinnamic acids:

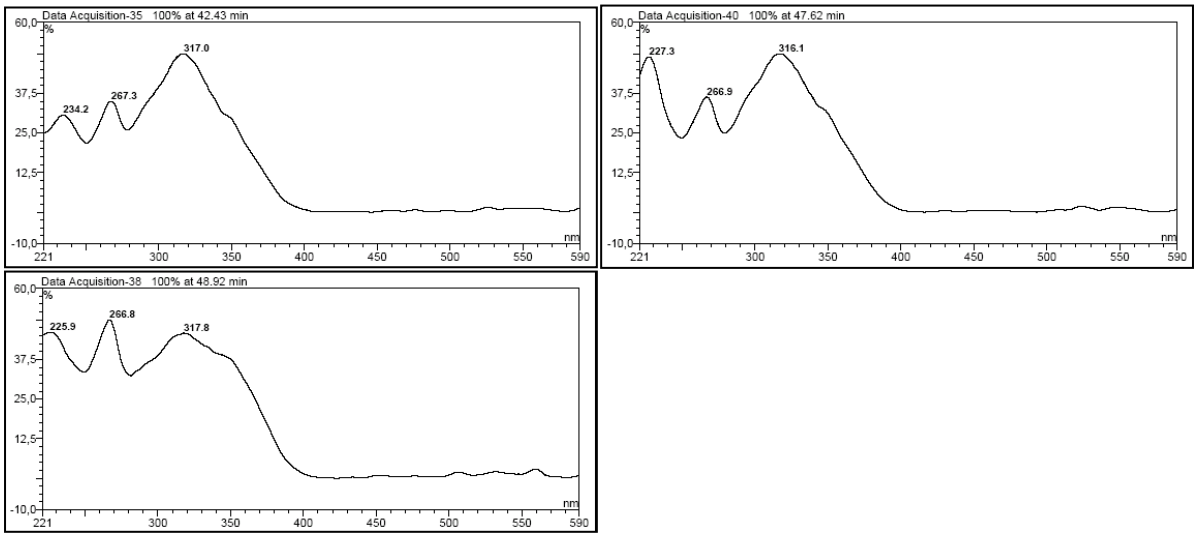


Fig. 8: UV spectra: Acylated kaempferol glycosides

Unidentified peaks

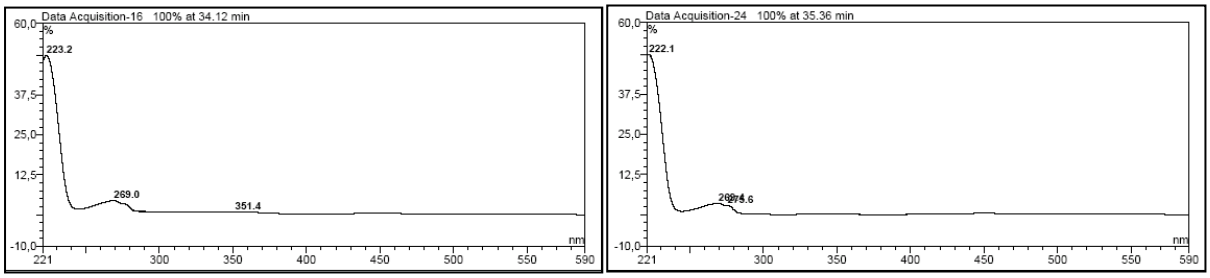
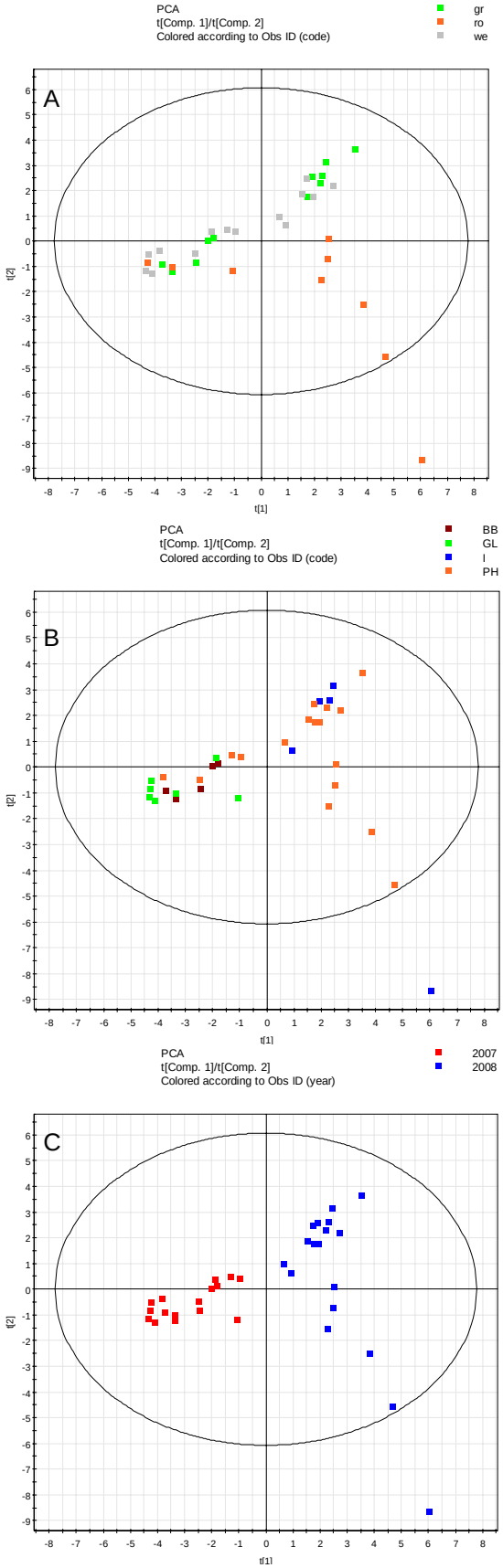


Fig 9: UV spectra: Unidentified phenols at 34.00 min and 35.00min

Statistics: PCA



gr… green outer tepals
ro… pink outer tepals
we… white outer tepals

BB… Bisamberg; NÖ; A
GL… St. Georgen; BGLD; A
I… Prematura; Istria; HR
PH… Perchtoldsdorfer Heide; W; A

Accessions 2007 and 2008

Fig. 10: PCA of all accessions

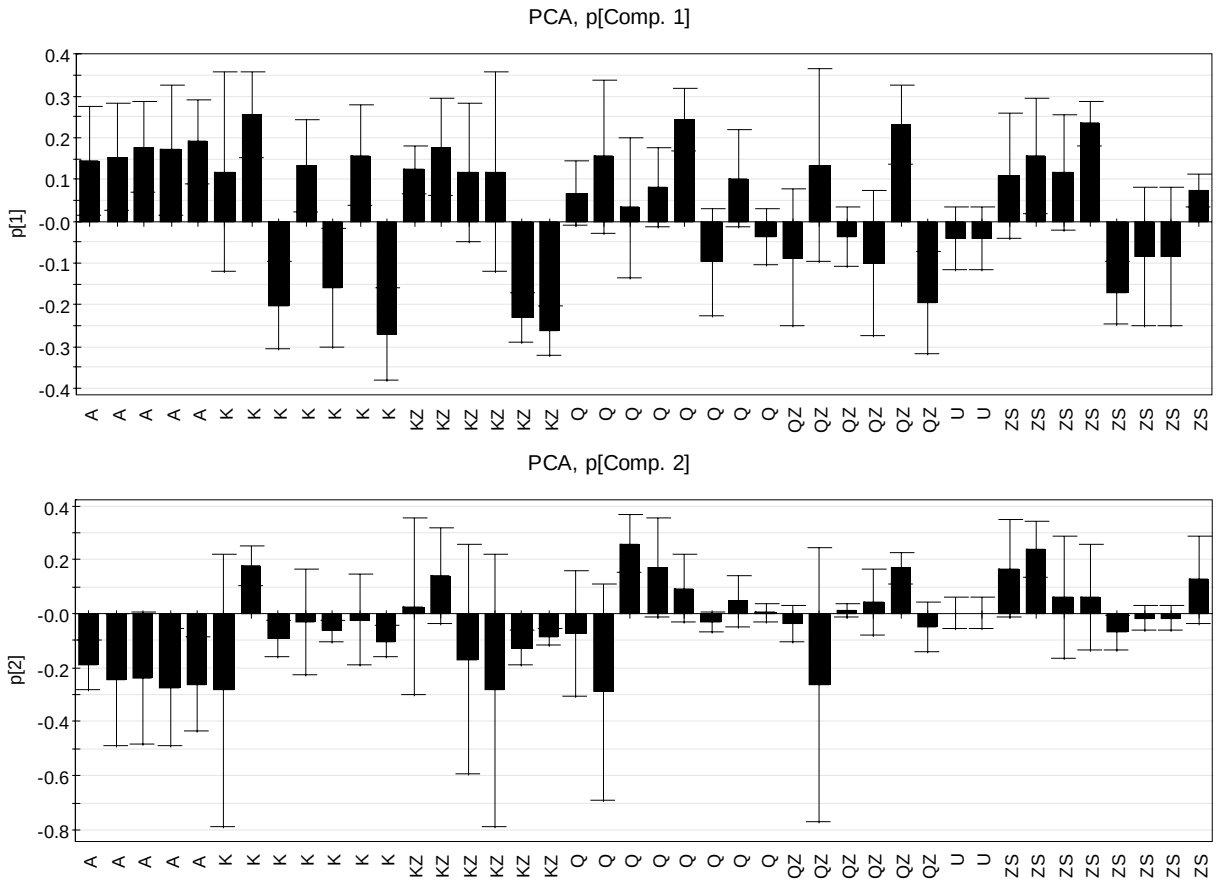


Fig. 11: Loading plots of variables, first and second principal component of PCA, A, anthocyan; K, kaempferol glycoside; KZ, acylated kaempferol glycoside; Q, quercetin glycoside; QZ, acylated quercetin glycoside; U, unknown phenol; ZS, cinnamic acid.

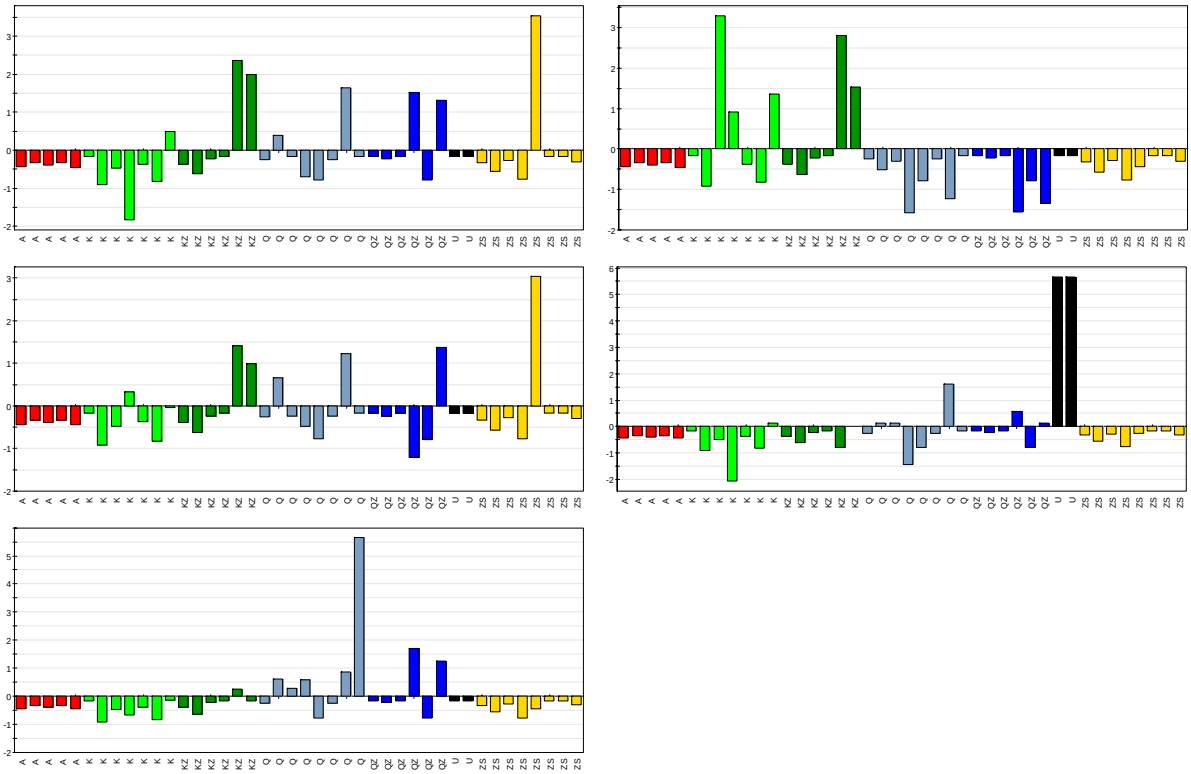


Fig. 12: *O. sphegodes*, green outer tepals, accessions 2007, Bisamberg, score contribution, PCA (observation – average); A, anthocyan; K, kaempferol glycoside; KZ, acylated kaempferol glycoside; Q, quercetin glycoside; QZ, acylated quercetin glycoside; U, unknown phenol; ZS, cinnamic acid.

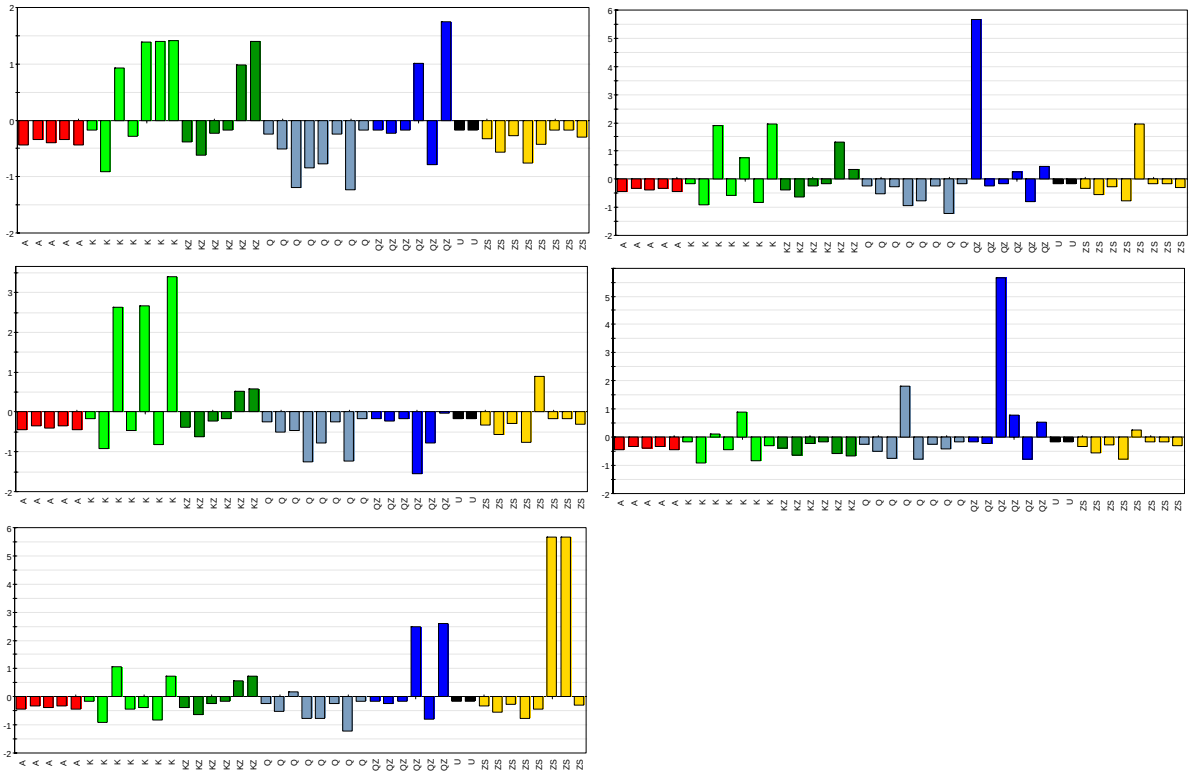


Fig. 13: *O. holoserica*; white outer tepals, accessions 2007, St. Georgen; score contribution PCA (observation – average); A, anthocyan; K, kaempferol glycoside; KZ, acylated kaempferol glycoside; Q, quercetin glycoside; QZ, acylated quercetin glycoside; U, unknown phenol; ZS, cinnamic acid.

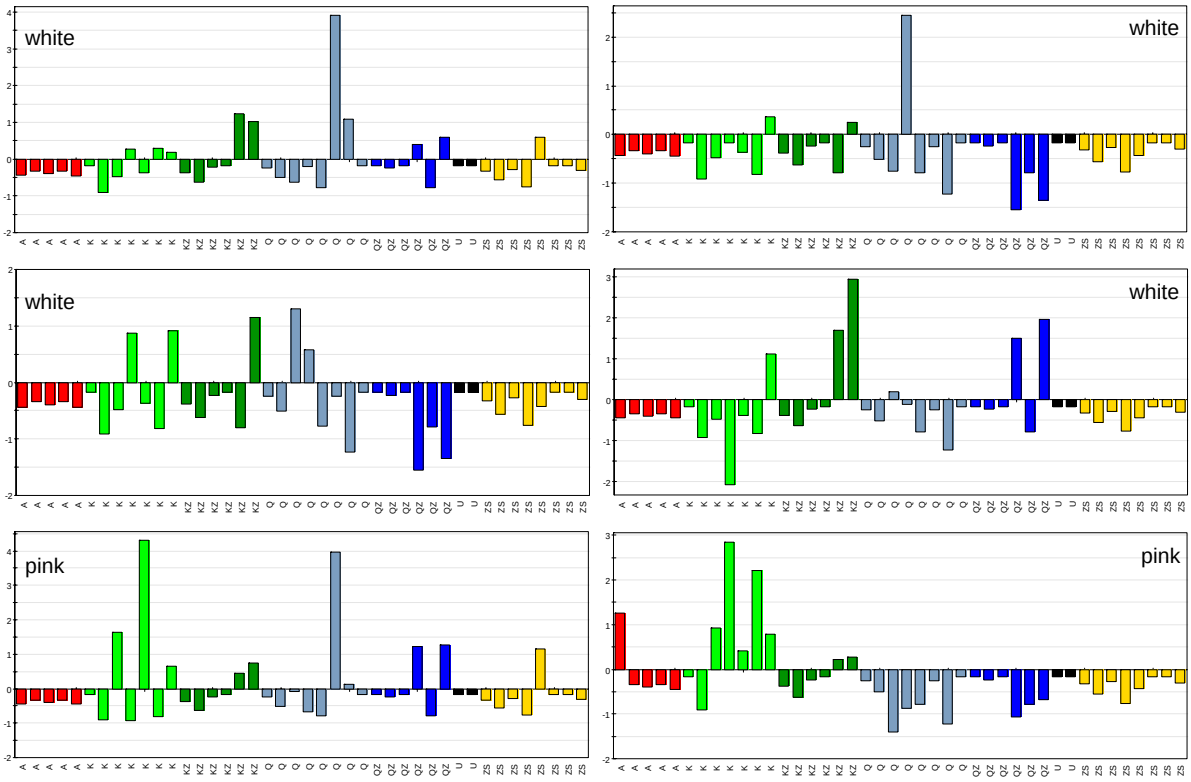


Fig. 14: *O. holoserica*; white and pink outer tepals, accession 2007, Perchtoldsdorfer Heide; Score contribution PCA (observation – average); A, anthocyan; K, kaempferol glycoside; KZ, acylated kaempferol glycoside; Q, quercetin glycoside; QZ, acylated quercetin glycoside; U, unknown phenol; ZS, cinnamic acid.

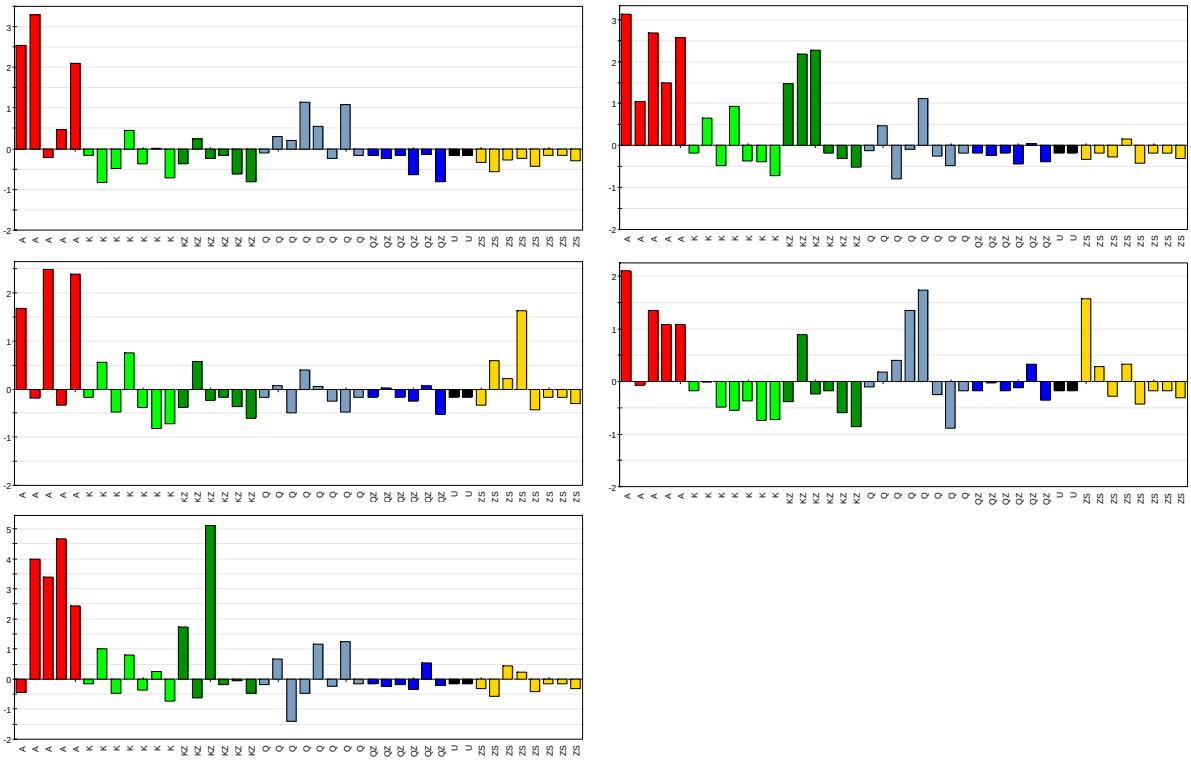


Fig. 15: *O. holoserica;* pink outer tepals, accessions 2008, Perchtoldsdorfer Heide; score contribution PCA (observation – average); A, anthocyan; K, kaempferol glycoside; KZ, acylated kaempferol glycoside; Q, quercetin glycoside; QZ, acylated quercetin glycoside; U, unknown phenol; ZS, cinnamic acid.

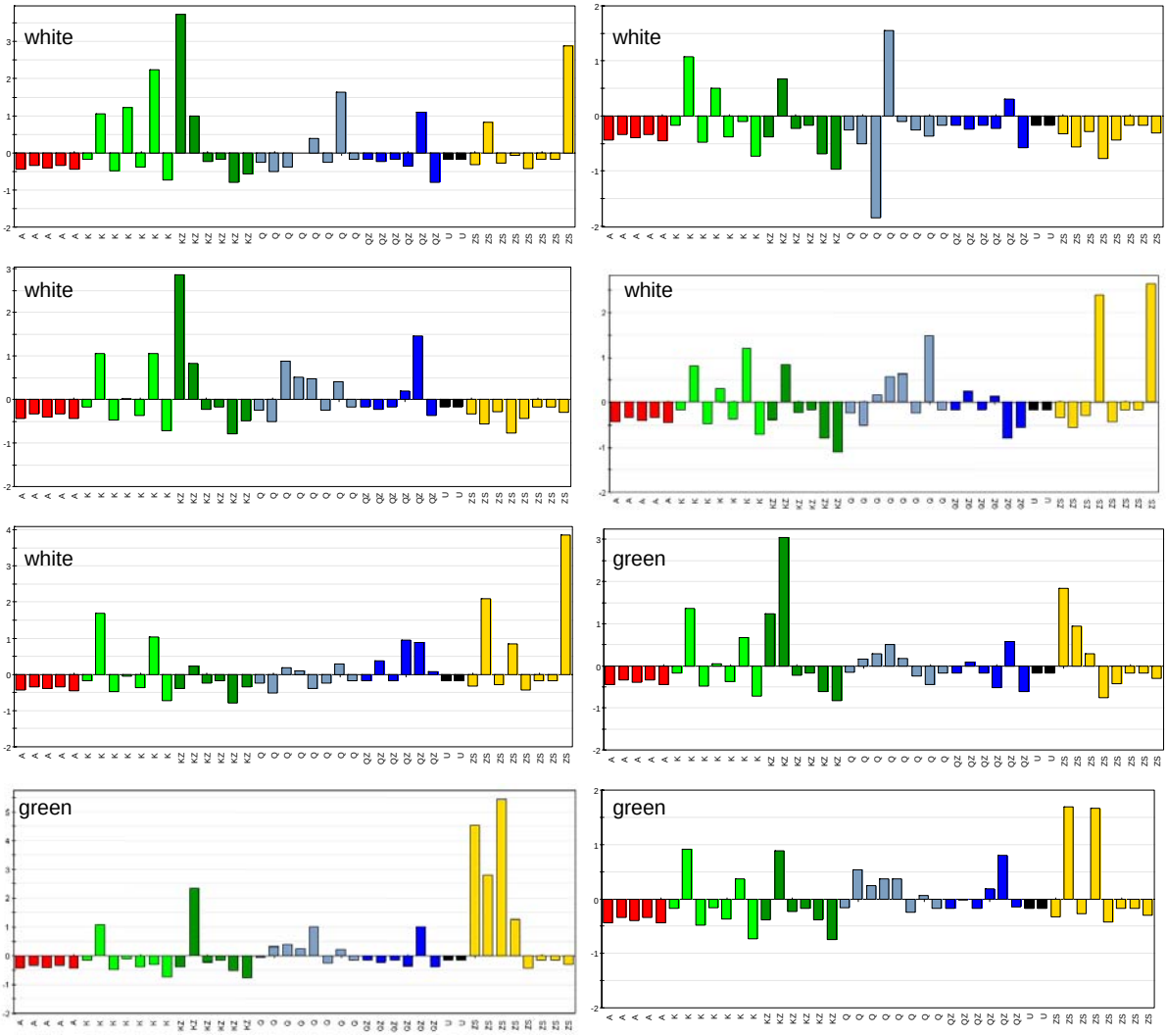


Fig. 16: *O. holoserica*; white and green outer tepals, accessions 2008, Perchtoldsdorfer Heide; score contributions PCA (observation – average); A, anthocyan; K, kaempferol glycoside; KZ, acylated kaempferol glycoside; Q, quercetin glycoside; QZ, acylated quercetin glycoside; U, unknown phenol; ZS, cinnamic acid.

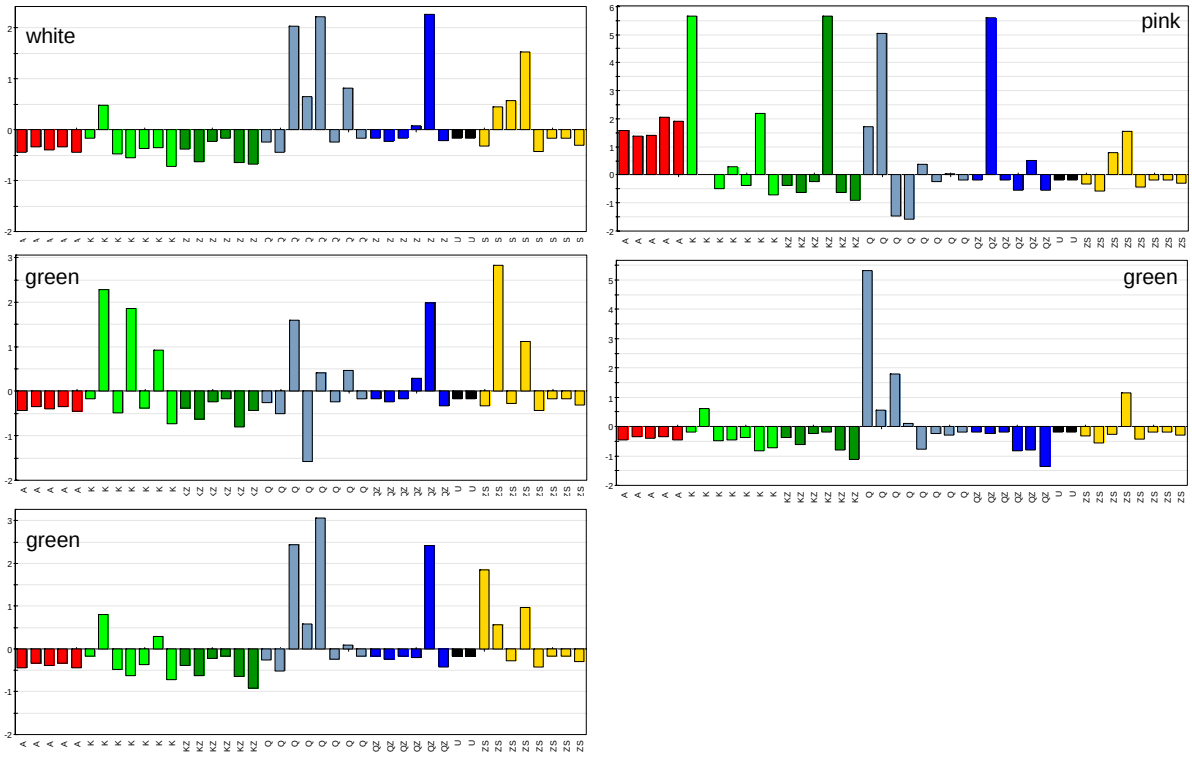


Fig. 17: *O. untchjii*; white, green and pink outer tepals, accessions 2008, Istria, Prematura; score contribution PCA (observation – average); A, anthocyan; K, kaempferol glycoside; KZ, acylated kaempferol glycoside; Q, quercetin glycoside; QZ, acylated quercetin glycoside; U, unknown phenol; ZS, cinnamic acid.

Differential pulse voltammetry

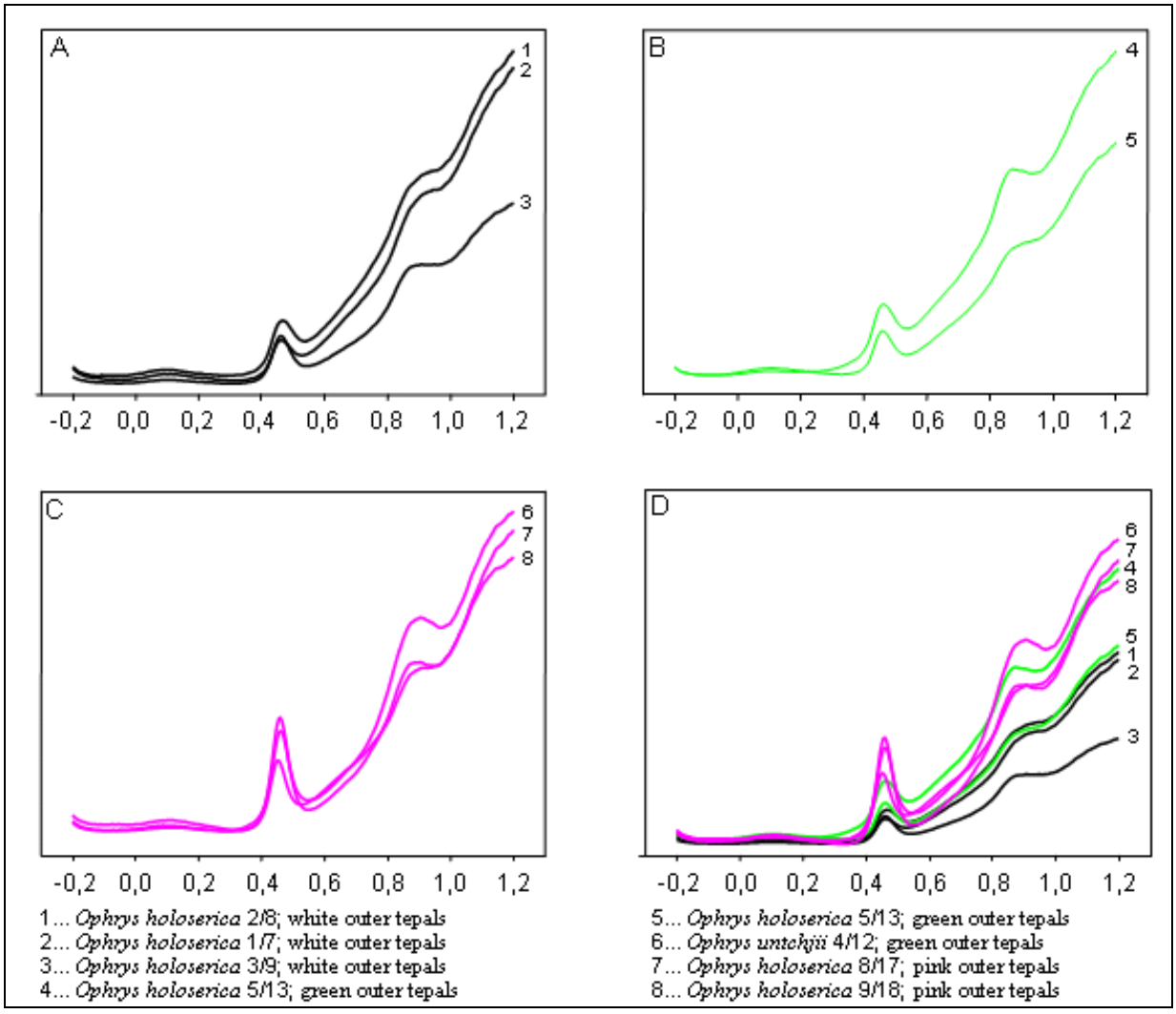


Fig. 18: Differential pulse voltammograms of differently coloured outer tepal extracts: White (A), green (B) and pink (C) outer tepals of *O. holoserica* and *O. untchjii*. All voltammograms were normalized to 5mg/ml.

Reflection spectroscopy

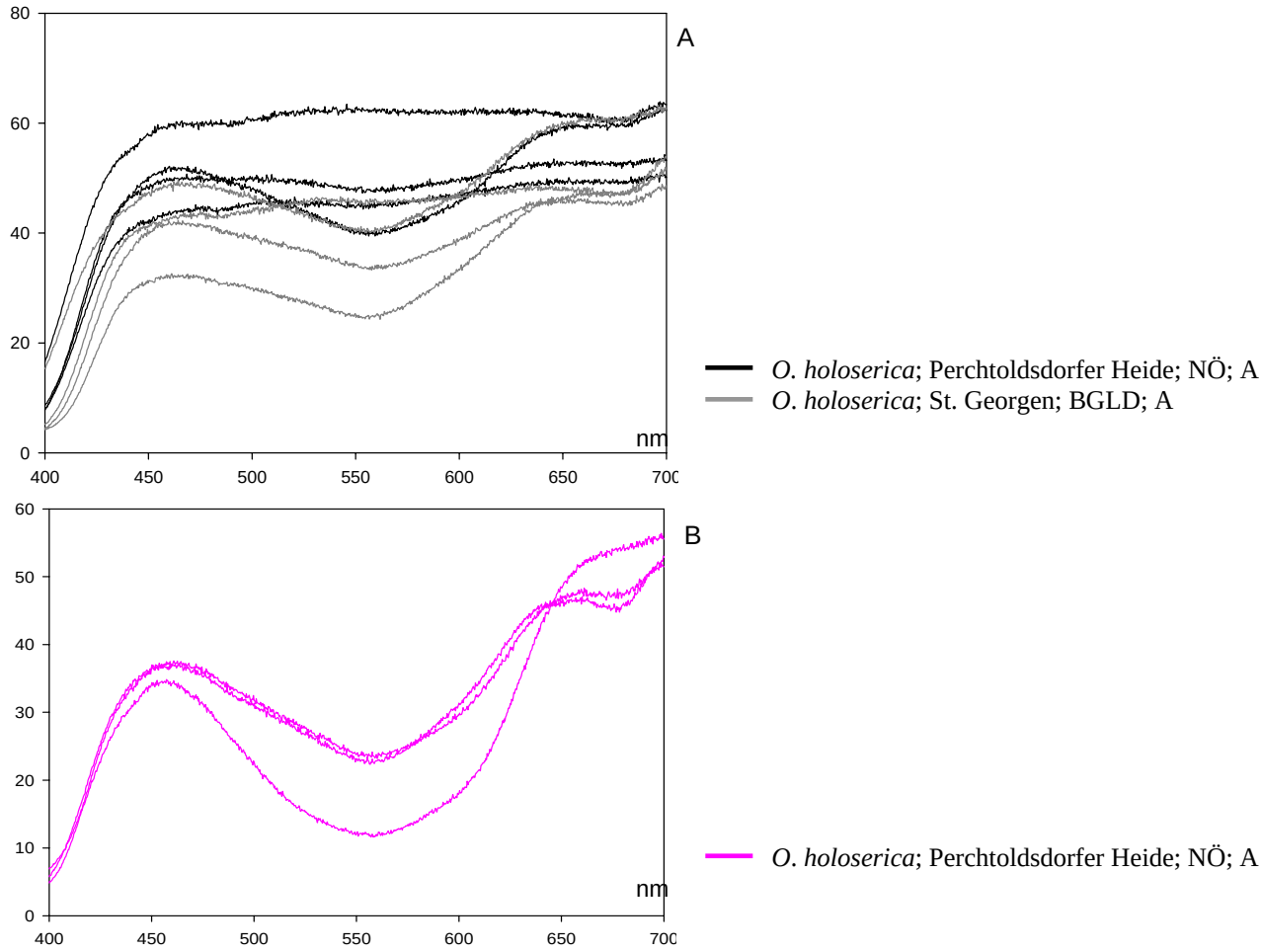


Fig. 19: Reflection spectroscopy: *O. holoserica*; accessions 2007; Perchtoldsdorfer Heide and St. Georgen; white outer tepals (A) and pink outer tepals (B)

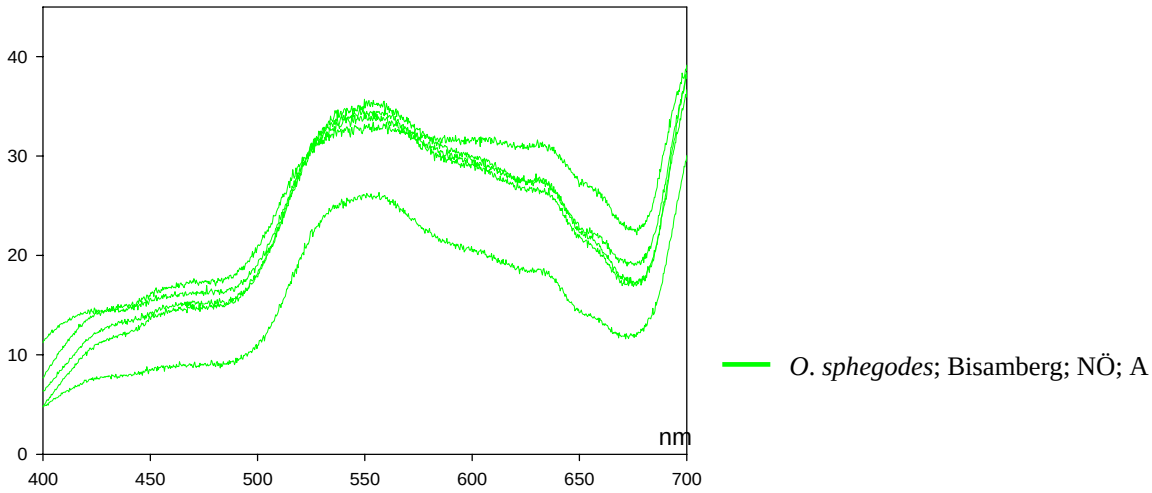


Fig. 20: Reflection spectroscopy: *O. sphegodes*; accessions 2007; Bisamberg; green outer tepals

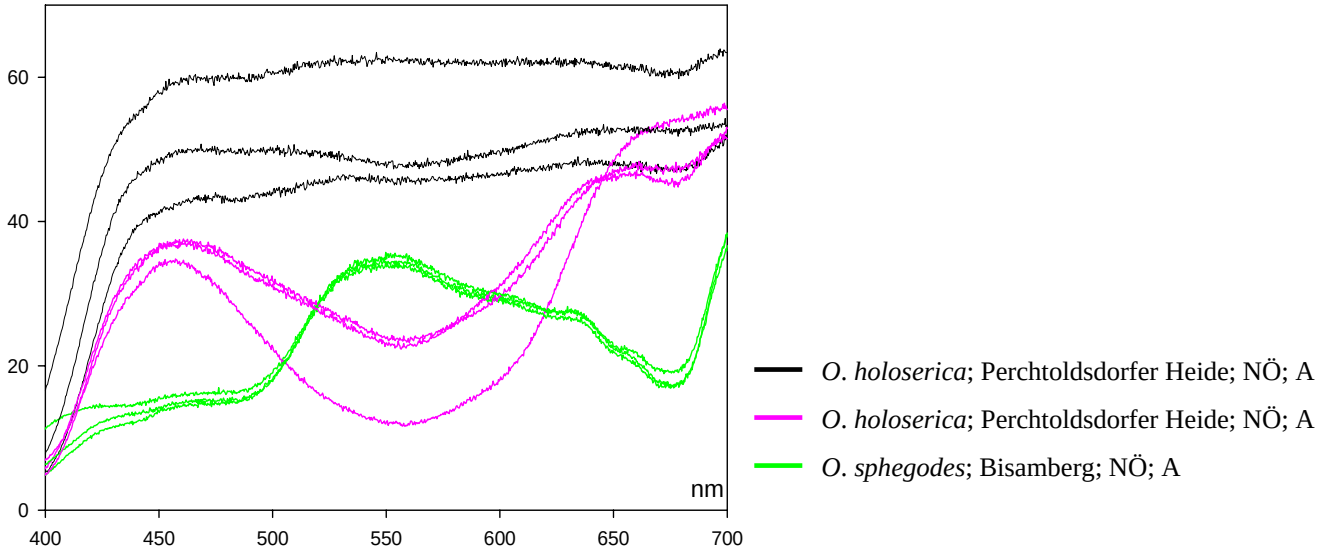


Fig. 21: Reflection spectroscopy: Overlay; *O. holoserica*, *O. sphegodes*; accessions 2007; white (black curves), green and pink outer tepals.

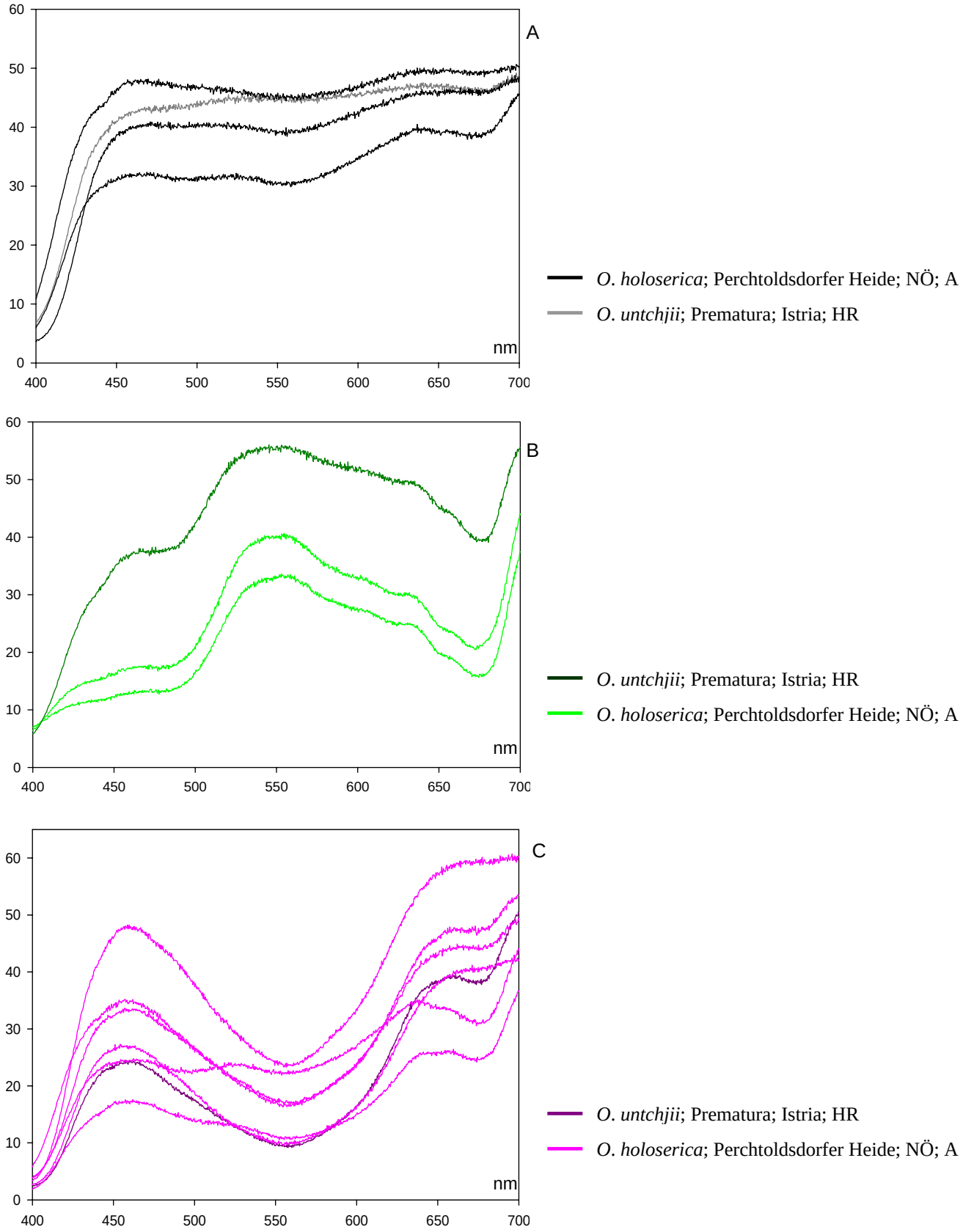


Fig. 22: Reflection spectroscopy; *O. holoserica*, *O. untchjii*, accessions 2008; white (A), green (B) and pink (C) outer tepals

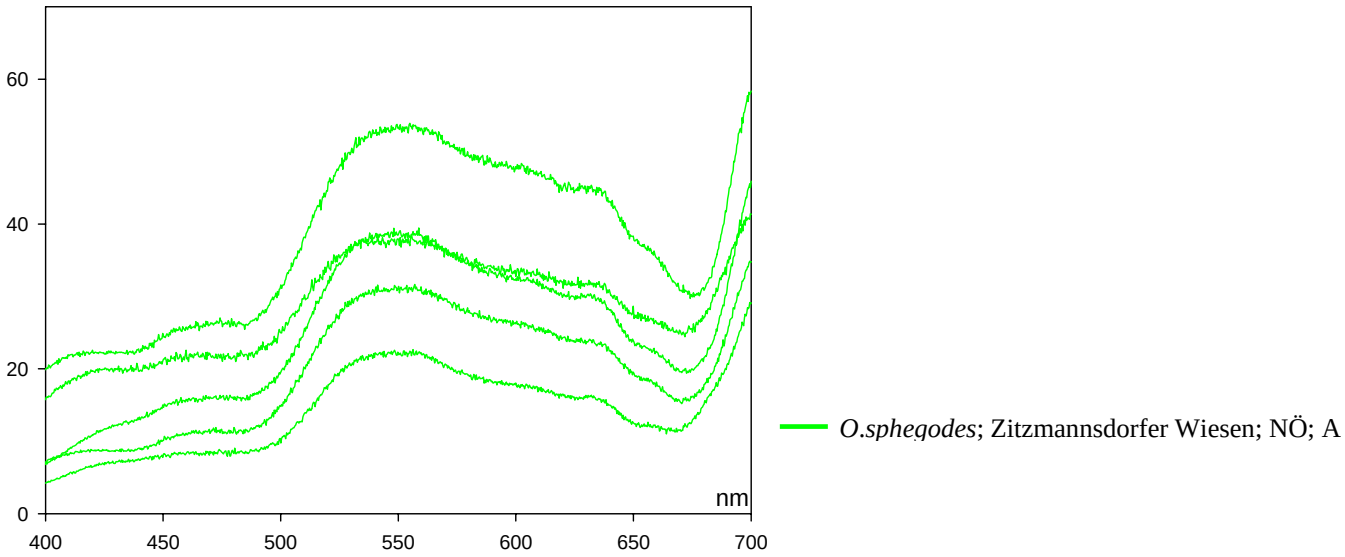


Fig. 23: Reflection spectroscopy: *O. sphegodes*, accessions 2008, green outer tepals (not analysed by HPLC)

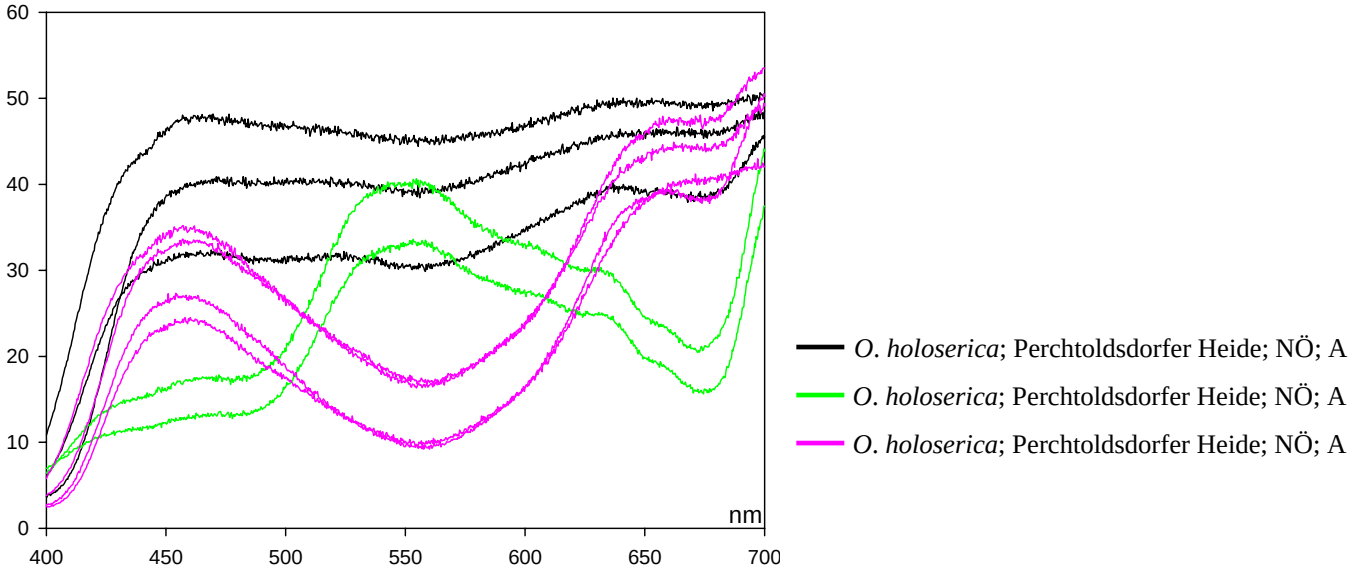
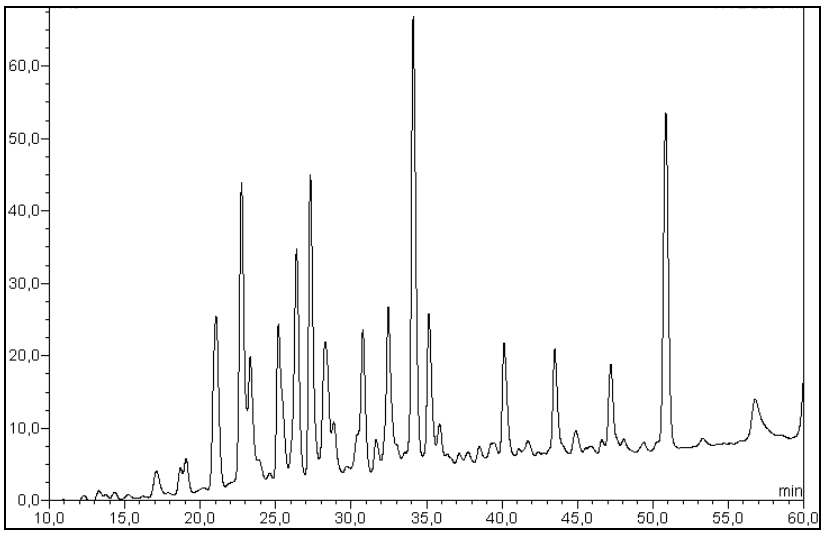


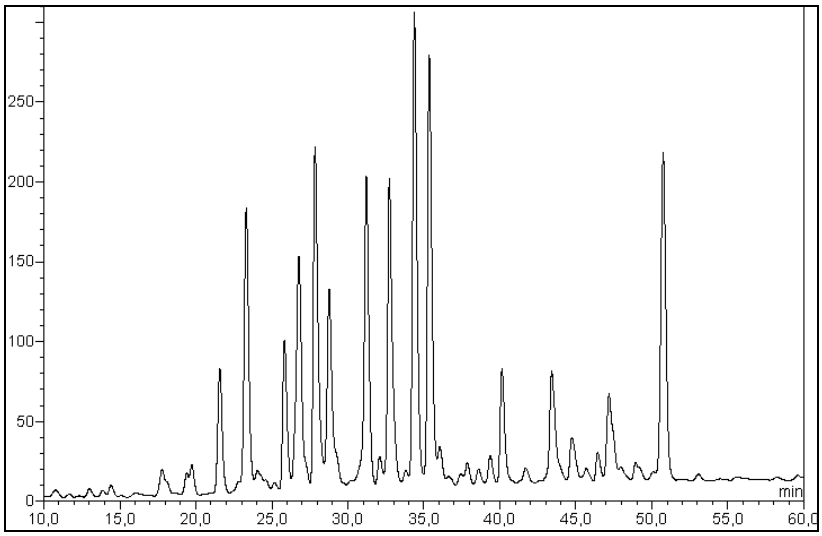
Fig. 24: Reflection spectroscopy: Overlay; *O. holoserica*, accessions 2008, white (black curves), green and pink outer tepals

Appendix

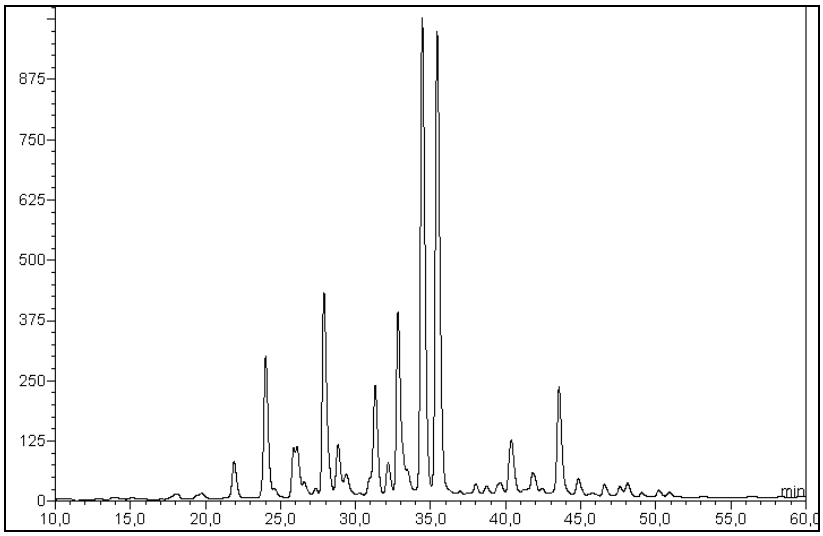
HPLC – analysis: *Ophrys*; outer tepal extracts, methanol/water (1/1; v/v), accessions 2008



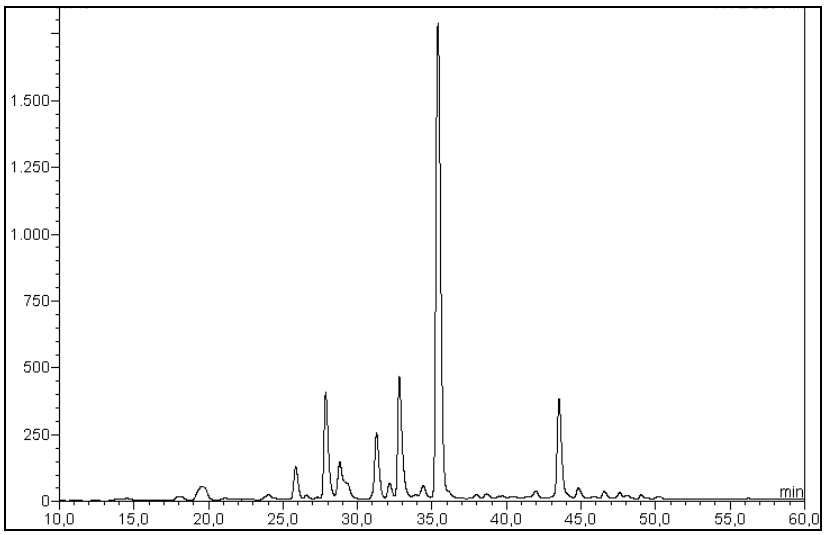
*O. untchjii*_1_white tepals; Prematura; Istria; HR



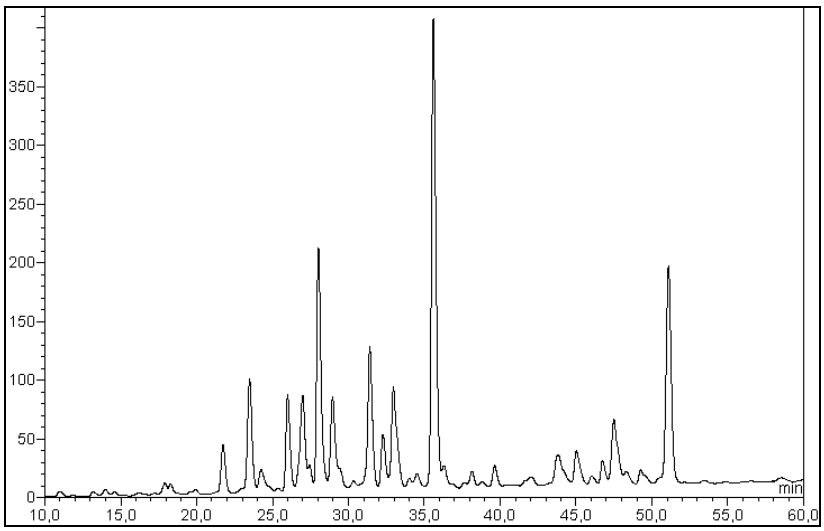
*O. untchjii*_2_green tepals; Prematura; Istria; HR



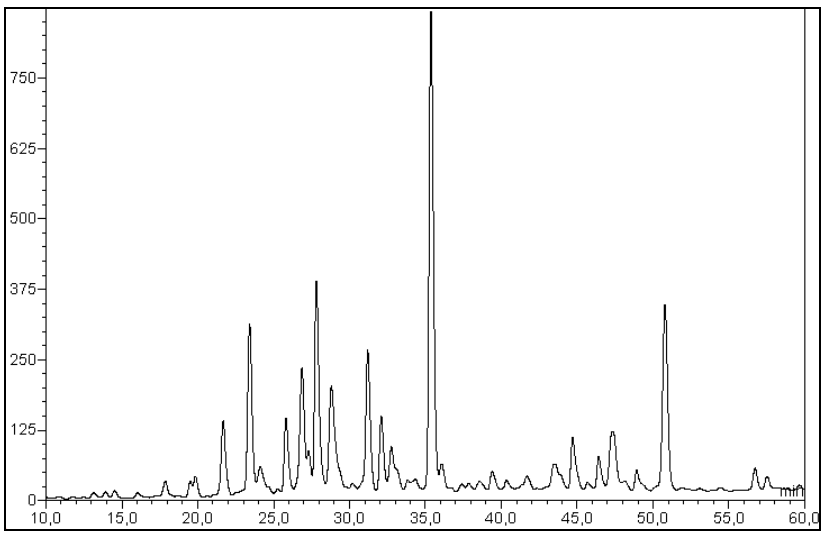
*O. untchjii*_3_pink tepals; Prematura; Istria; HR



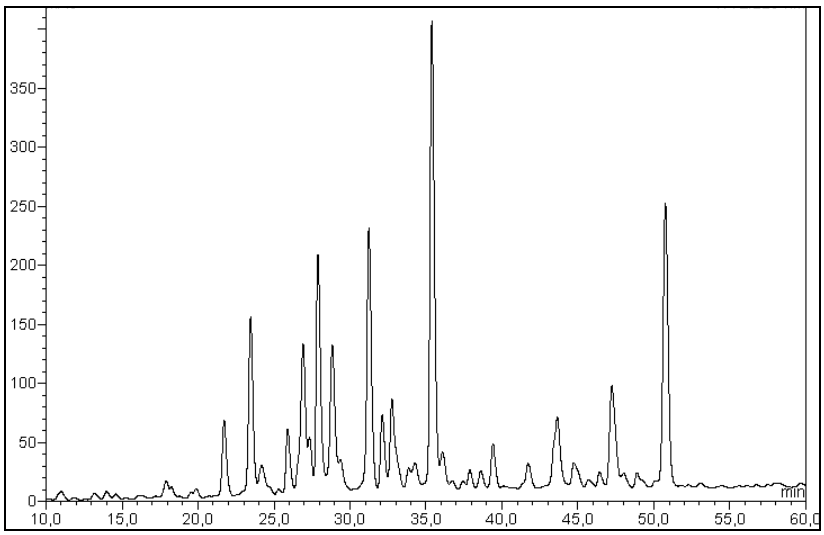
*O. holoserica*_5_green tepals
Perchtoldsdorfer Heide; W; A



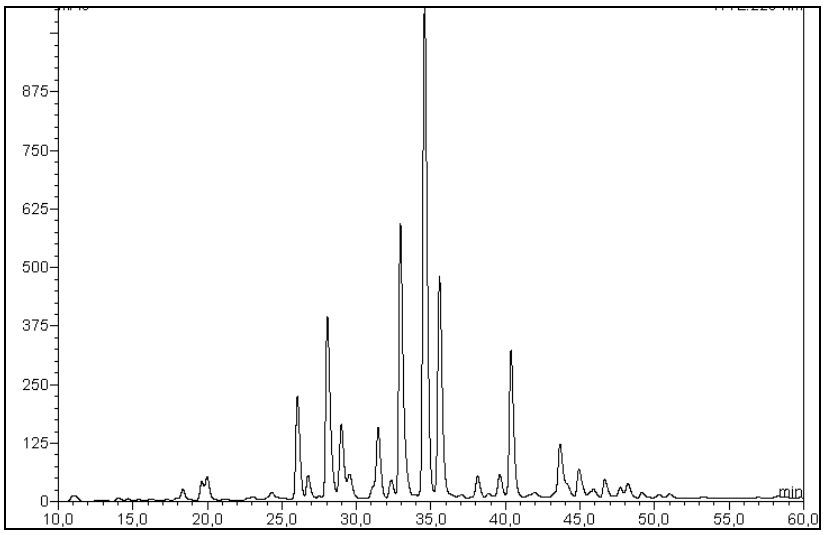
*O. holoserica*_7_white tepals;
Perchtoldsdorfer Heide; W; A



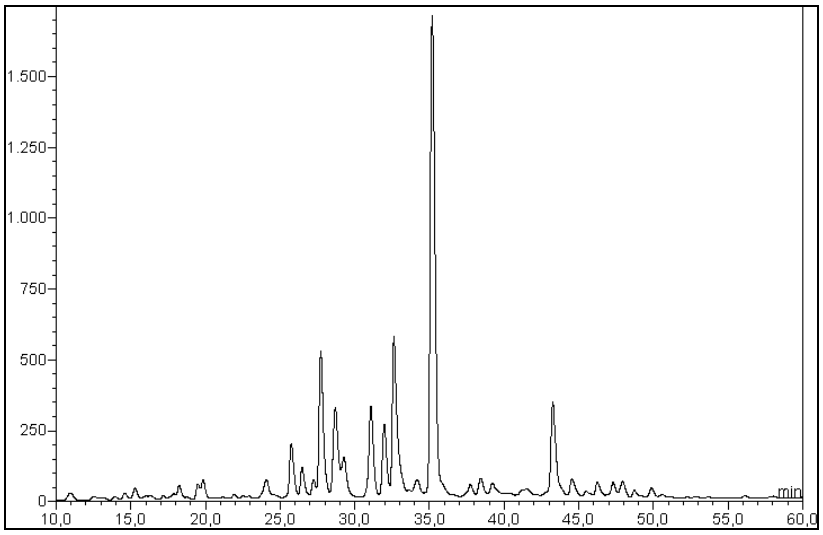
*O. holoserica*_8_white tepals;
Perchtoldsdorfer Heide; W; A



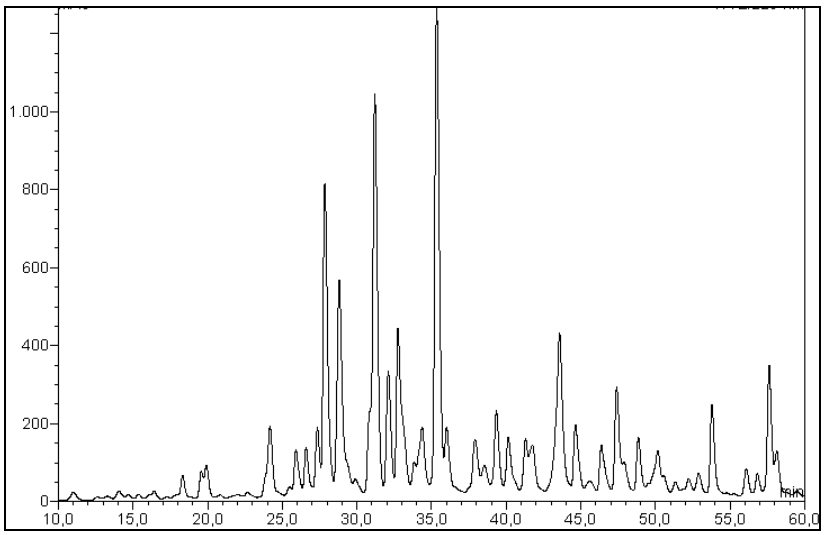
*O. holoserica*_10_white tepals; Perchtoldsdorfer Heide; W; A



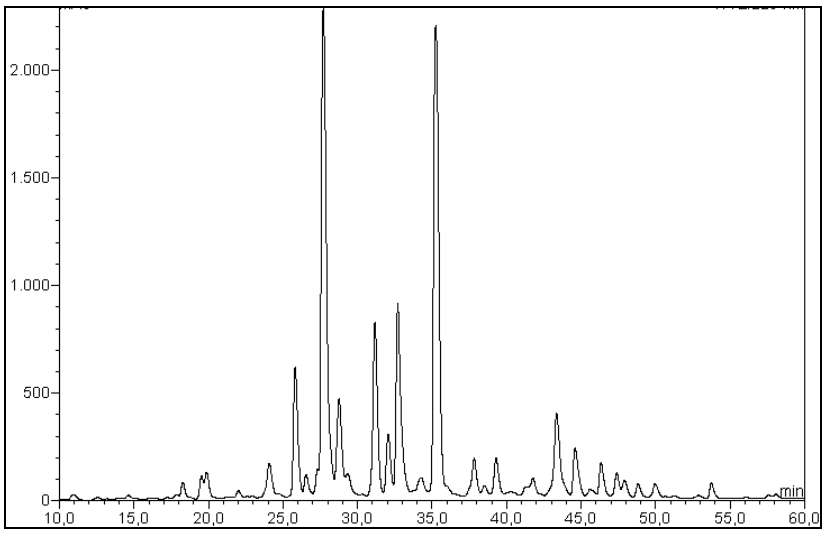
*O. untchjii*_12_green tepals; Prematura; Istria, HR;



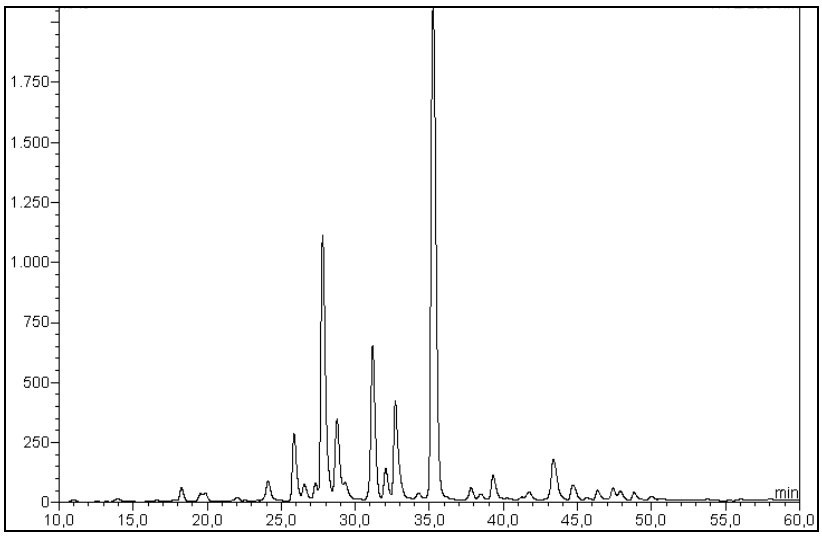
*O.holoserica*_13_green tepals; Perchtoldsdorfer Heide; W; A



*O. holoserica*_16_pink tepals; Perchtoldsdorfer Heide; W; A

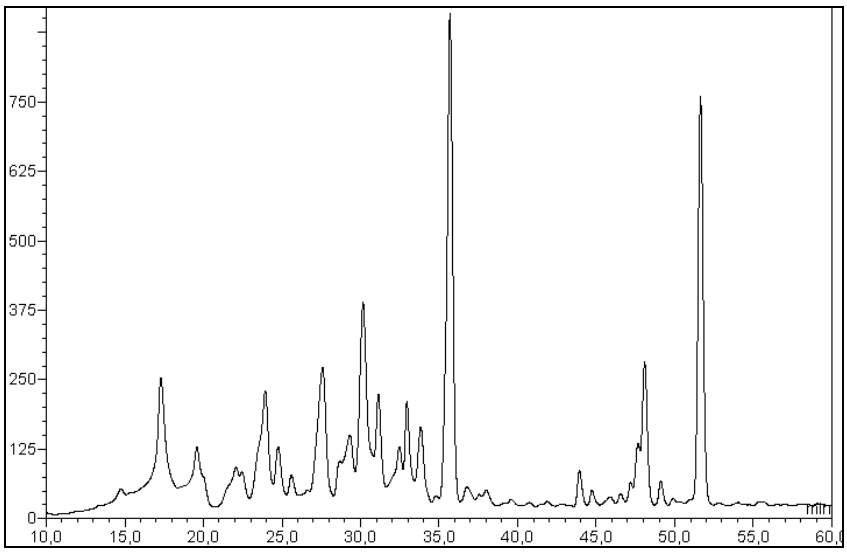


*O. holoserica*_17_pink tepals; Perchtoldsdorfer Heide; W; A

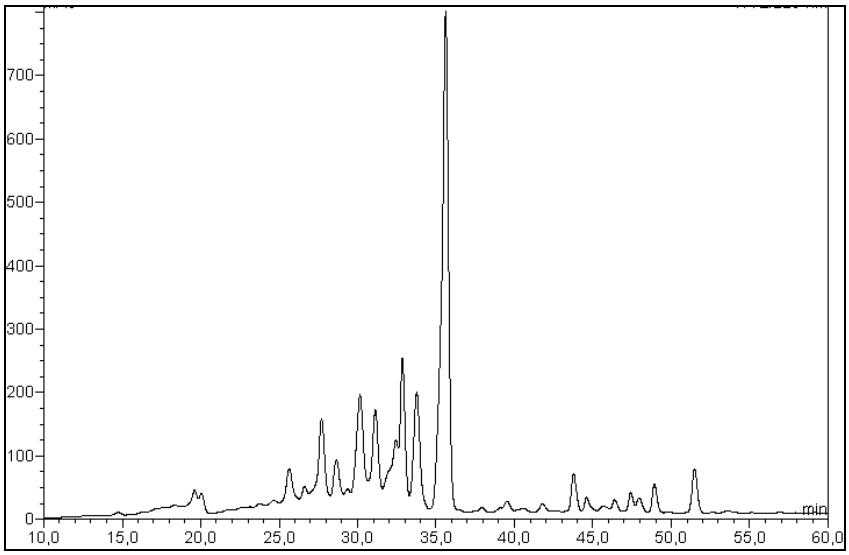


*O. holoserica*_18_pink tepals; Perchtoldsdorfer Heide; W; A

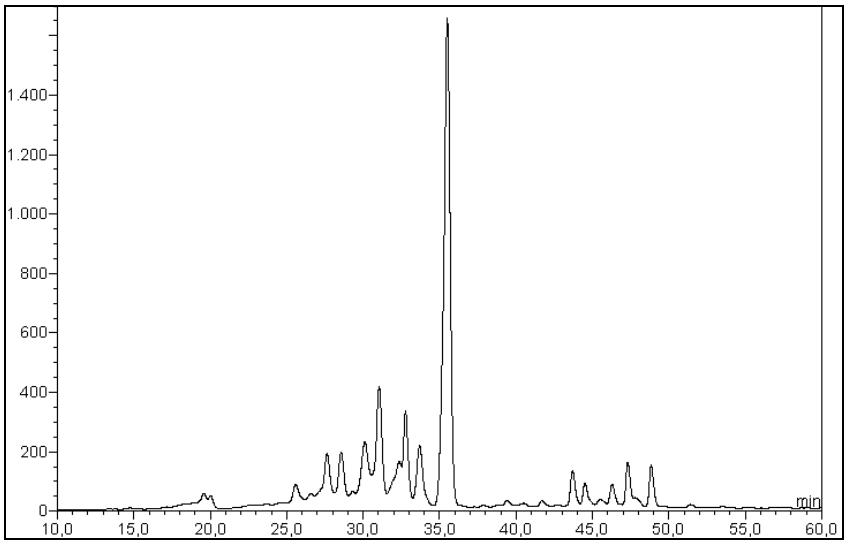
HPLC – analysis: *Ophrys*; ethanolic outer tepal extracts; accessions 2007



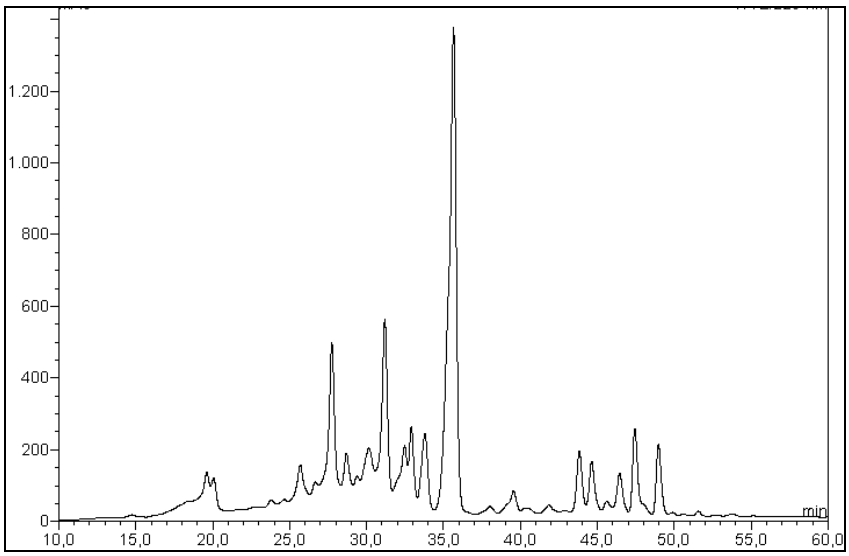
*O. holoserica*_1_white tepals; Perchtoldsdorfer Heide; W; A



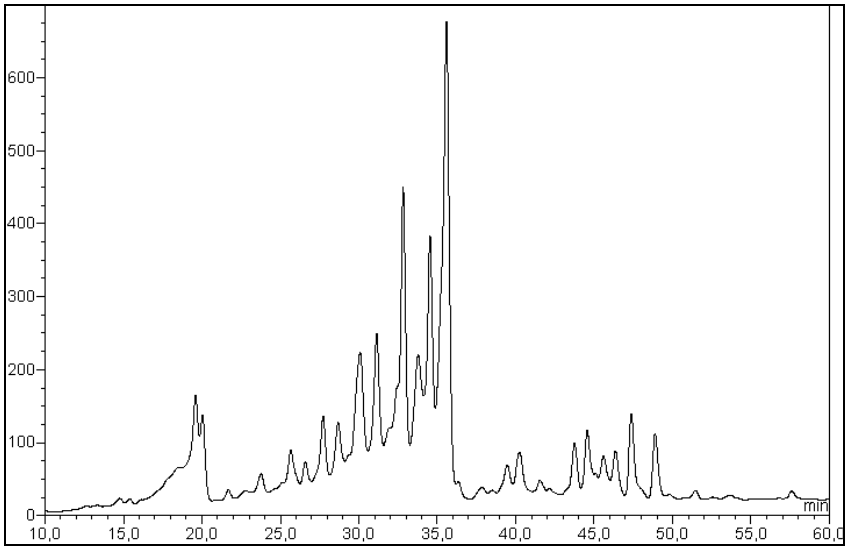
*O. holoserica*_2_white tepals; Perchtoldsdorfer Heide; W; A



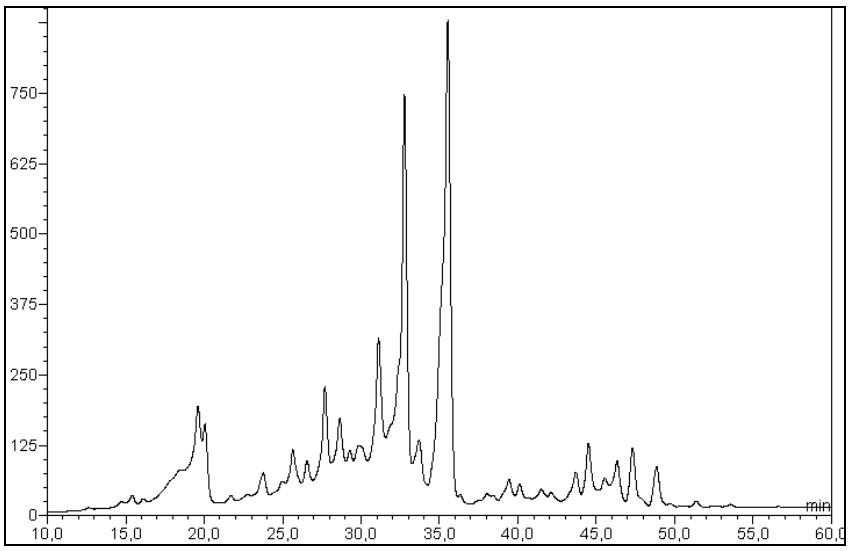
*O. holoserica*_3_white tepals; Perchtoldsdorfer Heide; W; A



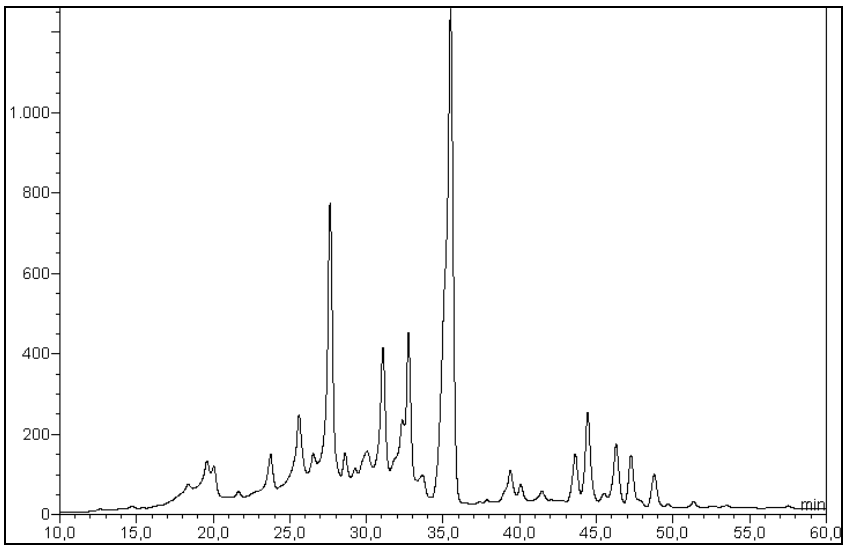
*O. holoserica*_4_white tepals
Perchtoldsdorfer Heide; W; A



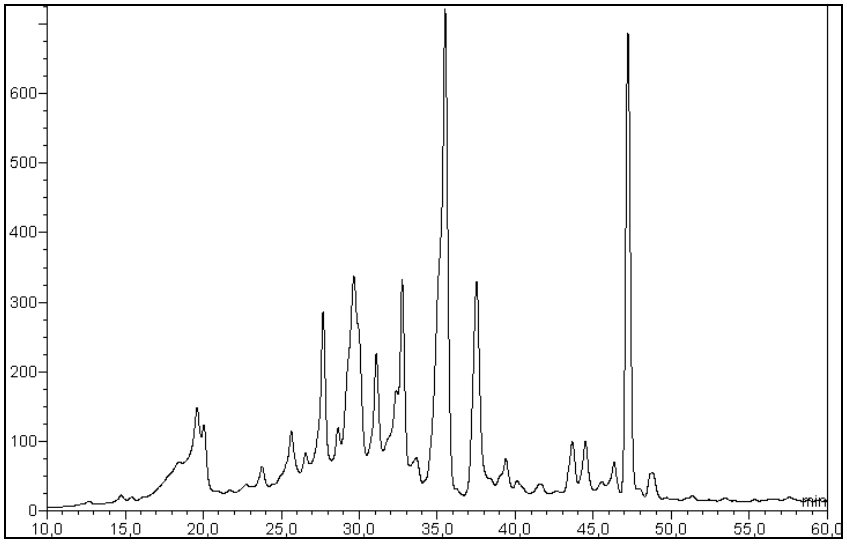
*O. sphegodes*_5_green tepals;
Bisamberg; NÖ; A



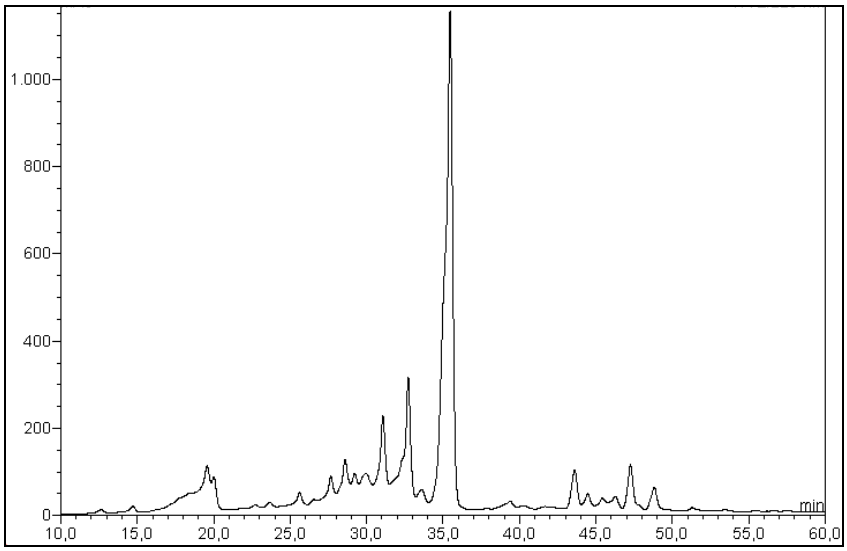
*O. sphegodes*_6_green tepals;
Bisamberg; NÖ; A



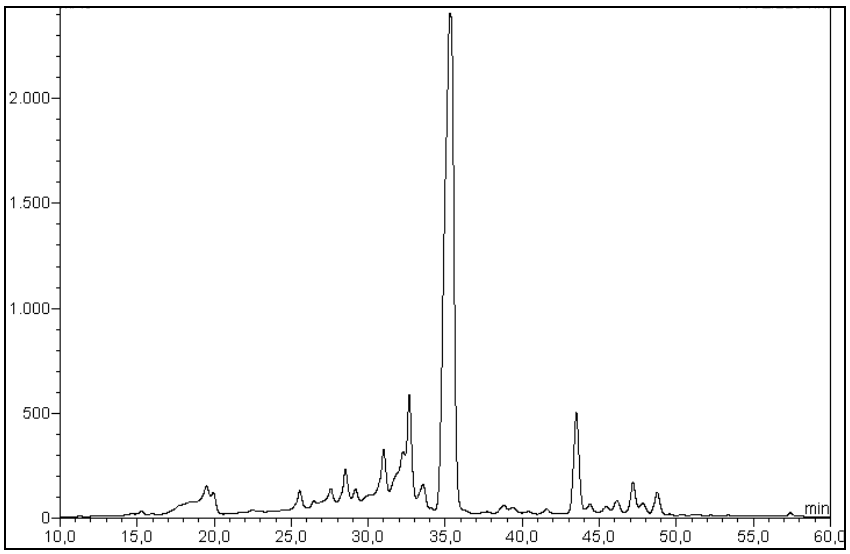
*O. sphegodes*_7_green tepals; Bisamberg; NÖ; A



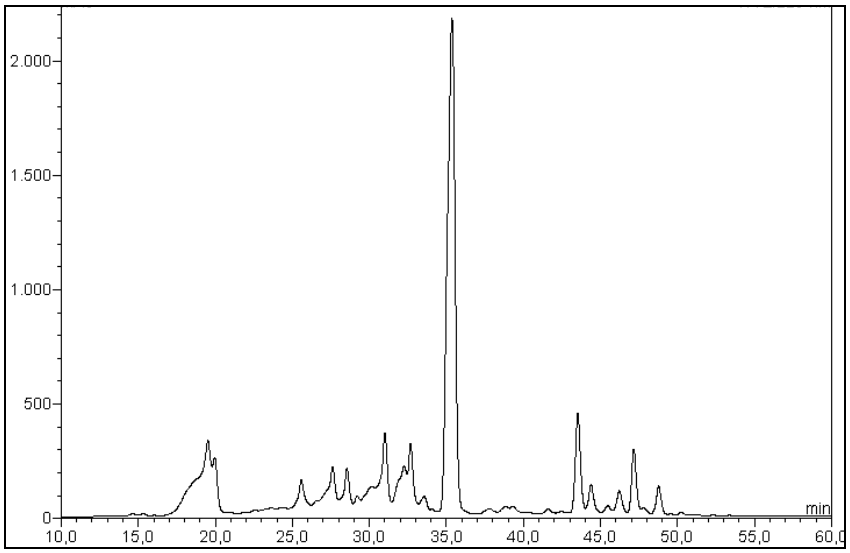
*O. sphegodes*_8_green tepals; Bisamberg; NÖ; A



*O. sphegodes*_9_green tepals; Bisamberg; NÖ; A



*O. holoserica*_10_white tepals; St.Georgen, BGLD; A



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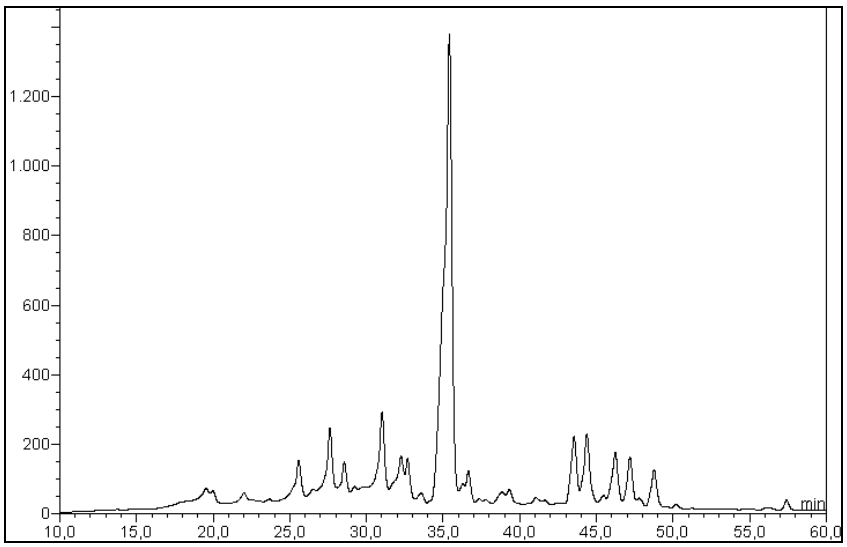
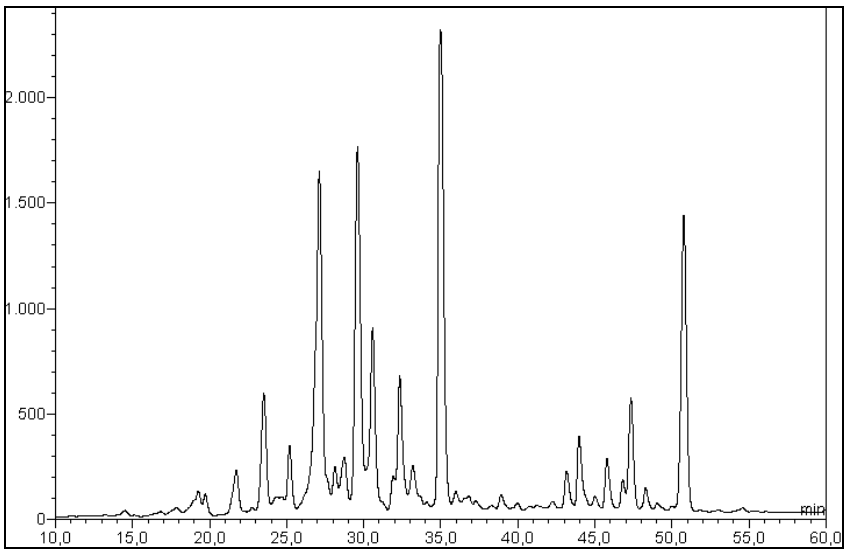
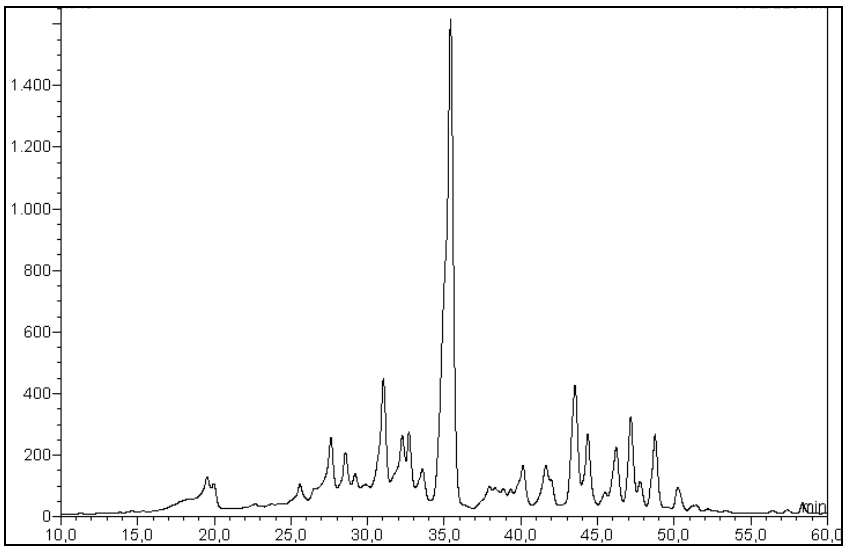


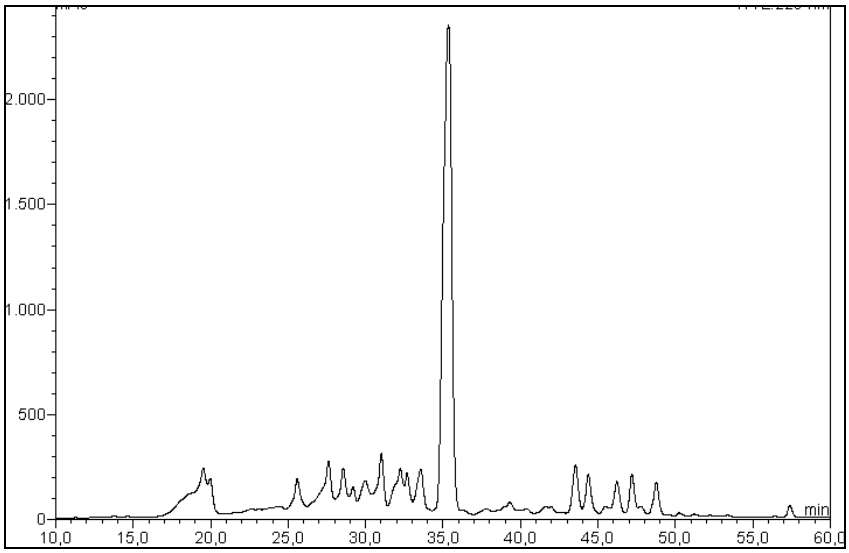
*O. holoserica*_12_white tepals; St.Georgen, BGLD; A



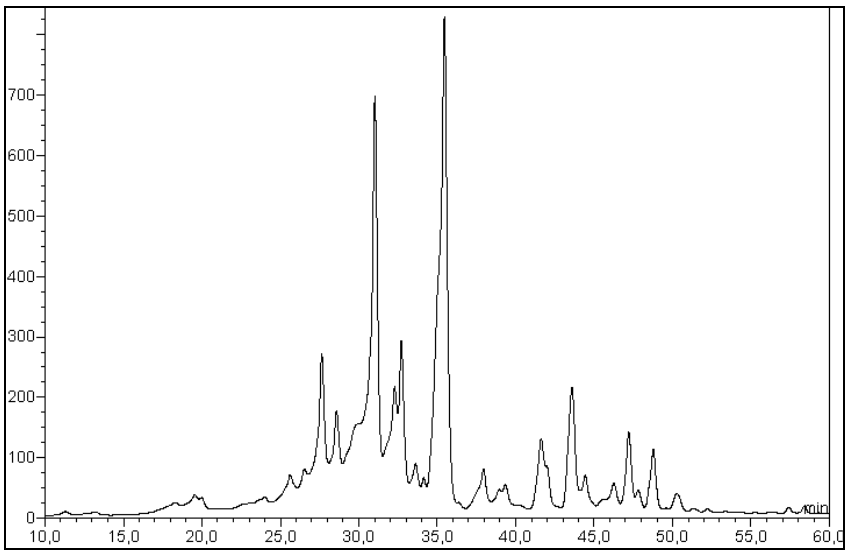
*O. holoserica*_13_white tepals; St.Georgen, BGLD; A



*O. holoserica*_14_pink tepals; Perchtoldsdorfer Heide; W; A

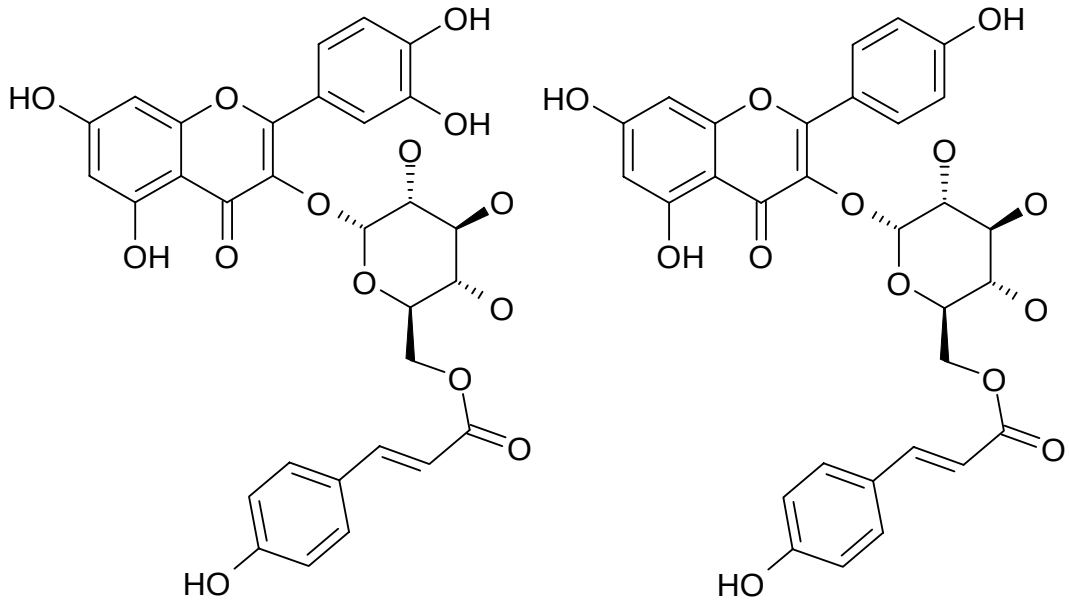


*O. holoserica*_15_pink tepals; Perchtoldsdorfer Heide; W; A



*O. holoserica*_16_pink tepals; Perchtoldsdorfer Heide; W; A

Acylated flavonoids



Possible structure for acylation of a quercetin -3-glycoside (see left) and kaempferol-3-glycoside (see right) with *p*-coumaric acid.

Zusammenfassung

In der Orchideengattung *Ophrys* zeigen *O. holoserica* und *O. untchii* drei unterschiedliche Färbungen der äußeren Tepalen, weiß, grün und pink. Mögliche Unterschiede in der Pigment Zusammensetzung, die individuelle antioxidative Aktivität in den unterschiedlich gefärbten Blütenblättern sowie ein möglicher Einfluss auf das Bestäuber Verhalten wurden in dieser Studie untersucht. 1% angesäuerte Methanolextrakte der äußeren Tepalen von *O. holoserica*, *O. untchjii* und grüne Tepalen von *O. sphegodes*, gesammelt an vier Standorten, wurden mit HPLC / DAD analysiert. HPLC – Profile wurden mit principle component analysis (PCA) verglichen und den jeweiligen Emissionsspektren der Tepalen gegenübergestellt. Eluierte Substanzen wurden mittels UV/ VIS -Detektion und Vergleich mit einer Spektrenbibliothek klassifiziert. Die antioxidative Aktivität wurde durch Differential Puls Voltammetrie (DPV) beziehungsweise Zyklischer Voltammetrie (CV) erfasst. Flavonoide, genauer die Flavonole Quercetin- und Kaempferolglykoside, stellten die vorherrschende Pigmentklasse in allen Extrakten dar. Anthocyane wurden ausschließlich in Extrakten der pink gefärbten Tepalen detektiert, allerdings nur in Spuren. UV Spektren einiger, in beinahe allen Messungen bemerkten Peaks deuteten auf die Anwesenheit acylierter Quercetin- und Kaempferol-Derivate hin. Zimtsäure Derivate wurden als höchstwahrscheinliche Acyl- Einheiten vermutet und überdies auch einzeln in fast allen Extrakten gefunden. Alle unterschiedlich gefärbten äußeren Tepalen zeigten antioxidative Aktivität, wobei sie bei pink gefärbten am Höchsten und bei weiß gefärbten am Niedrigsten zu beobachten war. Die Analyse durch PCA zeigte eine deutliche Gruppierung zwischen den Aufsammlungen der zwei verschiedenen Jahre, hervorgerufen durch unterschiedliche Umweltbedingungen. Das Frühjahr 2007 war durch lange Trockenperioden und wenig Niederschlag geprägt, während 2008 eine kühlere

Witterung und reichliche Niederschläge vorherrschten, was bessere Wachstumsbedingungen für Orchideen ermöglichte. Keine deutliche Gruppierung was hingegen zwischen den Pigment Komponenten der drei unterschiedlich gefärbten Tepalen, oder zwischen den vier Standorten zu beobachten. Eine leichte Gruppierung konnte nur bei pink gefärbten äußeren Tepalen feststellt werden, aufgrund der, nur in ihnen enthaltenen, Anthocyane. Verhaltensexperimente an den Bestäubern zeigten zwar eine Präferenz von Blüten mit pink gefärbtem äußeren Perigon, allerdings wurde dies als sekundäre Anlockung interpretiert. Ohne die Attraktivität durch emittierte Duftstoffe, den Sexpheromonen der weiblichen Individuen der Bestäuber, sind *Ophrys* Blüten für Bestäuber uninteressant. Dies deutete abermals auf eine primäre Funktion der Blütenpigmente als Schutz der Pflanze vor Stress wie etwa vor starker UV-Strahlung und Schutz durch ihre Aktivität als Radikalfänger und Antioxidantien.

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Curriculum vitae

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