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Mass spectrometric imaging of flavonoid glycosides and biflavonoids in *Ginkgo biloba* L.

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ABSTRACT

Ginkgo biloba L. is known to be rich in flavonoids and flavonoid glycosides. However, the distribution within specific plant organs (e.g. within leaves) is not known. By using HPLC-MS and MS/MS we have identified a number of previously known *G. biloba* flavonoid glycosides and biflavonoids from leaves. Namely, kaempferol, quercetin, isorhamnetin, myricetin, laricitrin/mearnsetin and apigenin glycosides were identified. Furthermore, biflavonoids like ginkgetin/isoginkgetin were also detected. The application of MALDI mass spectrometric imaging, enabled the compilation of concentration profiles of flavonoid glycosides and biflavonoids in *G. biloba* L. leaves. Both, flavonoid glycosides and biflavonoids show a distinct distribution in leaf thin sections of *G. biloba* L.

1. Introduction

Flavonoids and flavonoid glycosides are secondary plant metabolites present in plants (Habermehl et al., 2008). The functions include UV protection, pollen development, attraction of rhizobia, being antimicrobial and antifungal agents, chemical messengers, physiological regulators, cell cycle inhibitors, pigments to attract pollinators and seed distributors or feeding deterrents for plants (Fisher and Long, 1992; Galeotti et al., 2008; Jacobs and Rubery, 1988; Mo et al., 1992; Taylor and Grotewold, 2005; Winkel-Shirley, 2001). Additionally, plant extracts containing flavonoids and flavonoid glycosides have been used for thousands of years to treat and protect from various illnesses (Babu et al., 2013; Habermehl et al., 2008; Kleijnen and Knipschild, 1992; Manach et al., 2005; Ravishankar et al., 2013).

Ginkgo biloba L. is known to contain a vast number and variety of flavonoids in high concentrations (Kleijnen and Knipschild, 1992). These include myricetin, kaempferol, isorhamnetin, quercetin, rutin, laricitrin, mearnsetin, apigenin, luteolin, epicatechin, catechin and genistein (Dubber and Kanfer, 2004; Hasler et al., 1992; Liua et al., 2014; Pandey et al., 2014; van Beek, 2002). Furthermore, also biflavonoids like amentoflavone, bilobetin, isoginkgetin, ginkgetin and sciadopitysin are present in *G. biloba* L. leaf extracts (Yoshitama, 1997). Frequently, the aglyconic flavonoids are modified with glucose and rhamnose residues to form flavonoid glycosides (Hasler et al., 1992; Nasr et al., 1986; Victoire et al., 1988).

Analytics of flavonoid glycosides has so far been limited to analyses of contents and concentrations. While the distribution within the plant is barely known, the specific locations within a plant part or organ (e.g. within leaves) are not known at all. However, with the knowledge of such a specific distribution a correlation between structure and potential functions, indications towards synthesis or accumulation of different compounds in different cell types would be feasible. To investigate such a distribution, space-resolving techniques are demanded. With the recent developments in mass spectrometry (MS), the determination of the spatial resolution of substances in a tissue is now possible with high resolution. These techniques often use Desorption-Electrospray Ionization (DESI) or Matrix-Assisted Laser Desorption/Ionization (MALDI) as ionization method (Bodzon-Kulakowska and Suder, 2016). Especially the use of MALDI has driven bioanalytical sciences in recent years (Spengler, 2015). Here, we have optimized a workflow that enables identification and location specific detection of flavonoid glycosides and biflavonoids in order to generate mass spectrometric images of plant constituents from thin sections. The aim of this study is thus to investigate the distribution of flavonoid

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glycosides and biflavonoids in *G. biloba* leaf thin section to potentially enable further insights into the importance, biosynthesis and use of these substances within the plant.

2. Results & discussion

2.1. HPLC-MS and MS/MS of G. biloba leaf extracts

To investigate which flavonoids and flavonoid glycosides are present within the collected *G. biloba* L. leaves and to estimate the detectability by mass spectrometric methods, High Performance Liquid Chromatography (HPLC)-MS and MS/MS experiments were performed on methanolic leaf extracts. Table 1 shows the detected flavonoid glycosides. In order to identify specific glycosides, exact masses and fragmentation spectra were acquired. As previously described, flavonoid glycosides show a cleavage of sugar moieties upon fragmentation (Ma et al., 2001). Furthermore, flavonoids can be identified from their cross-ring fragmentation products and loss of small neutrals (Justino et al., 2009; Ma et al., 1997; Tsimogiannis et al., 2007). In our HPLC-MS/MS experiments sequential losses of a deoxyhexose (−146.0579 amu) and a hexose (−162.0528 amu) residue were already observed in survey spectra (Fig. 1). These two sugar moieties were attributed to rhamnose and glucose, as corresponding sugars have been found before. With the performed fragmentation experiments and the lack of corresponding standards, no further assignment of the linkage of containing sugars was possible (Satterfield and Brodbelt, 2001). Thus, the majority of flavonoids glycosides was present as *O*-rutinoside (aglycon-glucose(glc)-rhamnose(rha)) flavonoids. The presence of free flavonoid aglycones in HPLC-MS analyses and, thus, leaf extracts was excluded, as no corresponding chromatographic signals were observed. The only exception to this were apigenin containing compounds, as here two independent chromatographic signals were observed, stemming from the apigenin aglycon and the glucose modified apigenin, while no glucose and rhamnose modified apigenin diglycoside was detected.

Further, low resolution MS/MS experiments of the flavonoid aglycon signals from flavonoid glycosides in survey spectra allowed the identification of related structures. In summary, kaempferol-, quercetin-, isorhamnetin-, myricetin-, laricitrin-/mearnsetin- and apigenin-*O*-glycosides were found (Table 1, Fig. 2). No concluding decision could be made in case of laricitrin-/mearnsetin-*O*-glycosides, as no characteristic fragments were observed for either of the two. Additionally, in the fragmentation spectra of ginkgetin also signals originating from isoginkgetin were observed. This probably resulted from coelution of the two isomers. No quercitrin, catechin, epicatechin, genistein glycosides or free flavonoids nor apigenin diglycosides were detected in these experiments.

Table 1
Detected flavonoid glycosides and biflavonoids from *G. biloba* L. leaf extracts.

Flavonoid glycoside/ biflavones	Calculate sum formula[a]	R.T. [min]	*m/z* $[M + H]^+$ (observed)	*m/z* $[M + H]^+$ (calculated)	MS/MS verification of the flavonoid aglycones/biflavones[b]
Kaempferol-*O*-rutinoside	$C_{27}H_{30}O_{15}$	59.9	595.1649	595.1657	*m/z* 269 $[M + H\text{-}H_2O]^+$, *m/z* 259 $[M + H\text{-}CO]^+$, *m/z* 258 $[M + H\text{-}CHO]^+$, *m/z* 245 $[M + H\text{-}C_2H_2O]^+$, *m/z* 241 $[M + H\text{-}H_2O\text{-}CO]^+$, *m/z* 231 $[M + H\text{-}2CO]^+$, *m/z* 213 $[M + H\text{-}H_2O\text{-}2CO]^+$, *m/z* 165 $[^{0,2}A]^+$, *m/z* 153 $[^{1,3}A]^+$, *m/z* 147 $[M + H\text{-}2CO\text{-}C_4H_4O_2]^+$, *m/z* 137 $[^{0,2}A\text{-}CO]^+$, *m/z* 133 $[^{1,3}B\text{-}2H]^+$, *m/z* 121 $[^{0,2}B]^+$, *m/z* 111 $[^{1,3}A\text{-}C_2H_2O]^+$
Quercetin-*O*-rutinoside (Rutin)	$C_{27}H_{30}O_{16}$	54.2	611.1596	611.1607	*m/z* 285 $[M + H\text{-}H_2O]^+$, *m/z* 275 $[M + H\text{-}CO]^+$, *m/z* 274 $[M + H\text{-}CHO]^+$, *m/z* 257 $[M + H\text{-}H_2O\text{-}CO]^+$, *m/z* 247 $[M\text{-}2CO]^+$, *m/z* 229 $[M + H\text{-}H_2O\text{-}2CO]^+$, *m/z* 201 $[M + H\text{-}H_2O\text{-}3CO]^+$, *m/z* 195 $[^{0,4}B]^+$, *m/z* 165 $[^{0,2}A]^+$, *m/z* 153 $[^{1,3}A]^+$, *m/z* 149 $[^{1,3}B\text{-}2H]^+$, *m/z* 137 $[^{0,2}A\text{-}CO]^+/[^{0,2}B]^+$, *m/z* 111 $[^{1,3}A\text{-}C_2H_2O]^+$
Isorhamnetin-*O*-rutinoside	$C_{28}H_{32}O_{16}$	61.0	625.1753	625.1763	*m/z* 302 $[M + H\text{-}CH_3]^+$, *m/z* 299 $[M + H\text{-}H_2O]^+$, *m/z* 285 $[M + H\text{-}CH_3OH]^+$, *m/z* 271 $[M + H\text{-}H_2O\text{-}CO]^+$, *m/z* 261 $[M\text{-}2CO]^+$, *m/z* 257 $[M + H\text{-}CH_3OH\text{-}CO]^+$, *m/z* 243 $[M + H\text{-}H_2O\text{-}2CO]^+$, *m/z* 229 $[M + H\text{-}CH_3OH\text{-}2CO]^+$, *m/z* 201 $[M + H\text{-}CH_3OH\text{-}3CO]^+$, *m/z* 177 $[M + H\text{-}2CO\text{-}C_4H_4O_2]^+$, *m/z* 165 $[^{0,2}A]^+$, *m/z* 163 $[^{1,3}B\text{-}2H]^+$, *m/z* 153 $[^{1,3}A]^+$, *m/z* 151 $[^{0,2}B]^+$, *m/z* 111 $[^{1,3}A\text{-}C_2H_2O]^+$
Myricetin-*O*-rutinoside	$C_{27}H_{30}O_{17}$	47.5	627.1546	627.1556	*m/z* 301 $[M + H\text{-}H_2O]^+$, *m/z* 291 $[M + H\text{-}CO]^+$, *m/z* 290 $[M + H\text{-}CHO]^+$, *m/z* 283 $[M + H\text{-}2H_2O]^+$, *m/z* 273 $[M + H\text{-}H_2O\text{-}CO]^+$, *m/z* 263 $[M\text{-}2CO]^+$, *m/z* 255 $[M + H\text{-}2H_2O\text{-}CO]^+$, *m/z* 245 $[M + H\text{-}H_2O\text{-}2CO]^+$, *m/z* 195 *m/z* $[^{1,4}B]^+$, 193 $[^{0,4}B\text{-}H_2O]^+$, *m/z* 179 $[M + H\text{-}2CO\text{-}C_4H_4O_2]^+$, *m/z* 165 $[^{0,2}A]^+/[^{1,3}B\text{-}H_2O]^+$, *m/z* 153 $[^{1,3}A]^+/[^{0,2}B]^+$, *m/z* 137 $[^{0,2}A\text{-}CO]^+$, *m/z* 127 $[^{1,4}A{+}2H]^+$, *m/z* 111 $[^{1,3}A\text{-}C_2H_2O]^+$, *m/z* 109 $[^{0,4}A]^+$
Mearnsetin-/ Laricitrin-*O*-rutinoside [c]	$C_{28}H_{32}O_{17}$	55.9	641.1700	641.1712	*m/z* 318 $[M + H\text{-}CH_3]^+$, *m/z* 315 $[M + H\text{-}H_2O]^+$, *m/z* 301 $[M + H\text{-}CH_3OH]^+$, *m/z* 287 $[M + H\text{-}H_2O\text{-}CO]^+$, *m/z* 277 $[M + H\text{-}2CO]^+$, *m/z* 273 $[M + H\text{-}H_2O\text{-}CO]^+$, *m/z* 263 $[M\text{-}CH_3OH\text{-}CO]^+$, *m/z* 259 $[M + H\text{-}H_2O\text{-}2CO]^+$, *m/z* 255 $[M + H\text{-}2H_2O\text{-}CO]^+$, *m/z* 245 $[M + H\text{-}CH_3OH\text{-}2CO]^+$, *m/z* 207 $[^{0,4}B\text{-}H_2O]^+$, *m/z* 193 $[^{0,4}B\text{-}CH_3OH]^+$, *m/z* 179 $[^{1,3}B\text{-}2H]^+$, *m/z* 165 $[^{0,2}A]^+$, *m/z* 153 $[^{1,3}A]^+$, *m/z* 111 $[^{1,3}A\text{-}C_2H_2O]^+$, *m/z* 109 $[^{0,4}A]^+$
Apigenin-*O*-glycoside[d]	$C_{15}H_{10}O_5$ $C_{21}H_{20}O_{10}$	57.4 69.5	271.0601 433.1130	271.0601 433.1129	*m/z* 253 $[M + H\text{-}H_2O]^+$, *m/z* 243 $[M + H\text{-}CO]^+$, *m/z* 235 $[M + H\text{-}2H_2O]^+$, *m/z* 229 $[M + H\text{-}C_2H_2O]^+$, *m/z* 225 $[M + H\text{-}H_2O\text{-}CO]^+$, *m/z* 163 $[^{0,4}B]^+$, *m/z* 153 $[^{1,3}A]^+$, *m/z* 145 $[^{0,4}B\text{-}H_2O]^+$, *m/z* 135 $[^{1,3}A\text{-}H_2O]^+$, *m/z* 121 $[^{0,2}B]^+$, *m/z* 109 $[^{0,4}A]^+$
Amentoflavone	$C_{30}H_{18}O_{10}$	72.7	539.0972	539.0973	*m/z* 521 $[M + H\text{-}H_2O]^+$, *m/z* 497 $[M + H\text{-}C_2H_2O]^+$, *m/z* 479 $[M + H\text{-}CH_3OH\text{-}CO]^+$, *m/z* 465 $[M + H\text{-}H_2O\text{-}2CO]^+$, *m/z* 421 $[^{1,3}IIA]^+$, *m/z* 413 $[^{0,4}IB\text{-}H_2O]^+$, *m/z* 403 $[^{1,3}IIA\text{-}H_2O]^+$, *m/z* 387 $[^{1,3}IB]^+$, *m/z* 377 $[^{0,4}IIA]^+$
Bilobetin	$C_{31}H_{20}O_{10}$	73.6	553.1128	553.1129	*m/z* 535 $[M + H\text{-}H_2O]^+$, *m/z* 521 $[M + H\text{-}CH_3OH]^+$, *m/z* 511 $[M + H\text{-}C_2H_2O]^+$, *m/z* 435 $[^{1,3}IIA]^+$, *m/z* 401 $[^{1,3}IB]^+$, *m/z* 391 $[^{0,4}IIA]^+$
Ginkgetin/ Isoginkgetin [e]	$C_{32}H_{22}O_{10}$	75.9	567.1283	567.1286	*m/z* 552 $[M + H\text{-}CH_3]^+$, *m/z* 535 $[M + H\text{-}CH_3OH]^+$, *m/z* 525 $[M + H\text{-}C_2H_2O]^+$, *m/z* 521 $[M + H\text{-}H_2O\text{-}CO]^+$, *m/z* 449 $[^{1,3}IIA]^+$, (*m/z* 435 $[^{1,3}IIA]^+$), *m/z* 432 $[^{0,4}IB]^+$, (*m/z* 415 $[^{1,3}IB]^+$), *m/z* 401 $[^{1,3}IB]^+$
Sciadopitysin	$C_{33}H_{24}O_{10}$	79.0	581.1440	581.1442	*m/z* 566 $[M + H\text{-}CH_3]^+$, *m/z* 549 $[M + H\text{-}CH_3OH]^+$, *m/z* 539 $[M + H\text{-}C_2H_2O]^+$, *m/z* 535 $[M + H\text{-}H_2O\text{-}CO]^+$, *m/z* 449 $[^{1,3}IIA]^+$, *m/z* 431 $[^{1,3}IIA\text{-}H_2O]^+$, *m/z* 415 $[^{1,3}IB]^+$

[a] Sum formulae were calculated from the exact masses observed in HPLC-MS.
[b] Observed fragmentation in MS/MS spectra of flavonoid fragments/biflavonoids, fragment nomenclature according to [24].
[c] From the fragmentation spectra, no differentiation could be made for mearnsetin- or laricitrin-*O*-rutinoside.
[d] Apigenin was only detected as glucose glycoside and free flavone.
[e] *m/z* 435 and *m/z* 415 (in brackets), significant for isoginkgetin were also detected in MS/MS spectra of the ginkgetin chromatographic signal.

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