



High-performance thin-layer chromatography–multi-stage mass spectrometry methods for analyses of bee pollen botanically originating from sweet chestnut (*Castanea sativa* Mill.)

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Abstract

High-performance thin-layer chromatographic (HPTLC) silica gel and amino plates in combination with developing solvents containing formic and acetic acid were examined for HPTLC–multi-stage mass spectrometry (MSⁿ) analyses of chestnut bee pollen samples from Slovenia and Türkiye. Ethyl acetate–formic acid–acetic acid–water (10:1.1:1.1:2.6, V/V) and ethyl acetate–dichloromethane–formic acid–acetic acid (10:2.5:1:1.1, V/V) were used for development of silica gel and amino plates, respectively. Twofold pre-development was required for the developed HPTLC–MSⁿ methods. The first pre-development was performed with methanol–formic acid (10:3, V/V) for silica gel plates and methanol–formic acid (10:5, V/V) for amino plates. The second pre-development with methanol was equal for both types of the plates. Using the developed HPTLC–MSⁿ methods, five phenylamides (spermidines), six isorhamnetin glycosides and gluconic acid were identified in both chestnut bee pollen samples. Glycosylated phenolic acid (caffeic acid-hexoside) was detected only in the Turkish bee pollen sample. To the best of our knowledge, this is the first report on isorhamnetin-(hexosyl)hexoside, isorhamnetin-acetylhexoside, isorhamnetin-(pentosyl-deoxyhexosyl)hexoside and caffeic acid-hexoside in chestnut bee pollen. This is also the first report on isorhamnetin-(pentosyl-deoxyhexosyl)hexoside and caffeic acid-hexoside in any bee products.

Keywords Thin-layer chromatography (TLC) · Chromatography · Tandem mass spectrometry · Fragmentation · Polyphenols · Flavonoids

1 Introduction

Sweet chestnut (*Castanea sativa* Mill.), which has been used as a timber and fruit tree since the Neolithic Age, is a deciduous and monoecious tree species with lanceolate leaves. The female flowers are located at the base of the male flowers grouped in 5–15 cm long catkins [1] that produce nectar and pollen. Pollen is spread by the wind or by insects such as honey bees (*Apis mellifera* L.). Young bee

workers consume protein-rich pollen for the development of the mandibular glands (salivary glands) and the hypopharyngeal glands, which produce royal jelly – food for the developing larvae and the bee queen [2]. Chestnut pollen belongs to those types of pollen that stimulate the development of hypopharyngeal glands [3]. Pollen also affects larval weight, immunity, glucose and lipid levels, etc. [3]. Due to phenolic compounds, bee pollen has antioxidant [4–7], anti-inflammatory [5] and antimicrobial [5, 7] properties. Chestnut bee pollen could safely be included in the human diet as a daily food supplement because it inhibits oxidative stress [6].

On the list of phenolic compounds found in chestnut bee pollen by targeted high-performance liquid chromatography (HPLC) analyses are: phenolic acids (gallic acid, syringic acid [8], rosmarinic acid [9, 10], protocatechuic acid [9]), flavanone (pinocembrin [8–10]), flavones (chrysin [8, 10], apigenin [9], apigenin-8-*C*-glucoside [9, 10] and luteolin [10]) and flavonols (galangin [8, 9], kaempferol [8], isorhamnetin [8] and quercetin-3-*O*-galactoside [8–10]). The only report on non-targeted chromatographic analyses in

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chestnut bee pollen, in which phenolic compounds (flavanone naringenin, glycosides of flavonol isorhamnetin) and phenylamides were discovered, was published recently [11]. Phenylamides (hydroxycinnamic acid amides) are amides conjugated with hydroxycinnamic acids such as caffeic, ferulic, coumaric, and sinapic acid [11].

High-performance thin-layer chromatography (HPTLC) was mainly used in the analysis of bee pollen samples for fingerprinting [4, 5, 12] with chemometric evaluation [12] and bioautography [4], in the past, also for testing the purity of isolates [13]. HPTLC separations of phenolic compounds in bee pollen samples were performed on silica gel plates [4, 5, 12] developed with developing solvents containing formic acid [5, 12] or formic acid and acetic acid [4, 5]. When coupling HPTLC and mass spectrometry (MS) via elution head-based interface, acids in the developing solvent cause intense background signals of clusters (e.g. sodium formate clusters [14–19]) or even sets of clusters [16] in mass spectra obtained using electrospray ionisation (ESI). These clusters disable MS detection, as they cause strong ion suppression of the desired compounds [14–19]. Ion suppression can be overcome by twofold pre-development (first methanol–formic acid, second solvent for elution of the desired chromatographic zones from the plate) of the silica gel plate [14, 16–19]. This protocol enabled the detection of phenolic acids [16, 18, 20], flavonoids [14, 16, 18, 20–22], anthraquinones [23] and physalins [17, 19] in various extracts from different plant [14–23] and food [16, 18] materials when a developing solvent contains only formic acid in addition to organic solvents.

The aims of this study were: (1) to develop solvent mixtures for pre-development of HPTLC silica gel and amino (NH_2) plates developed with solvent mixtures containing formic and acetic acid at the same time, to eliminate or at least significantly reduce the intensity of the background signals of acids based clusters in mass spectra; (2) to use the developed protocols for the first online HPTLC–multi-stage mass spectrometry (MS^n) methods for non-targeted analyses of phenolic compounds on HPTLC silica gel and NH_2 plates; and (3) to apply the developed HPTLC– MS^n methods for the analyses of phenolic compounds in chestnut bee pollen samples from Slovenia and Türkiye.

2 Experimental

2.1 Chemicals

All solvents were at least of analytical grade. Methanol (HPLC and LC–MS grade) was from Honeywell Reagents (Seelze, Germany). Ethyl acetate, acetic acid (glacial, 100%) and formic acid (98–100%) were from Merck (Darmstadt, Germany). Diphenylboric acid 2-aminoethyl ester was from

Sigma-Aldrich (Steinheim, Germany) and polyethylene glycol 4000 (PEG) was from Fluka Chemie (Buchs, Switzerland). Ultrapure water was obtained by a Milli-Q water purification system ($18 \text{ M}\Omega \text{ cm}^{-1}$) (Millipore, Bedford, MA, USA).

2.2 Bee pollen samples

Bee pollen samples were collected by professional beekeepers from their hives in Kranj (Slovenia – SI) and Artvin (Türkiye – TR). The palynological analyses were performed according to the published methodology [24]. The analysed bee pollen samples from Kranj and Artvin contained 98.0% and 97.0% sweet chestnut (*Castanea sativa* Mill.) pollen, respectively, and were identified as chestnut bee pollen samples based on the classification of Barth [25]. The samples were stored at -20°C .

2.3 Sample test solutions

The bee pollen samples were pulverised using the Micro-Dismembrator S (Sartorius, Göttingen, Germany) at a frequency of 1700 min^{-1} for 1 min. Pulverised bee pollen samples (500 mg) were dispersed in 80% (V/V) ethanol–water (5 mL). After 30 min of ultrasound-assisted extraction, the suspensions were centrifuged at 4200 rpm for 5 min and filtered through a $0.45 \mu\text{m}$ polyvinylidene difluoride (PVDF) syringe filter (Macherey–Nagel, Düren, Germany) into storage vials to obtain sample test solutions [STSs (100 mg/mL): chestnut bee pollen test solutions from Slovenia (STS–SI) and Türkiye (STS–TR)]. All STSs were stored at -20°C prior to analysis. Immediately prior to analysis, the STSs were transferred to 2 mL vials.

2.4 HPTLC– MS^n analyses

For HPTLC–MS/MS and HPTLC– MS^n analyses, $20 \text{ cm} \times 10 \text{ cm}$ glass-backed HPTLC silica gel 60 (art. no. 1.05641, Merck) and glass-backed HPTLC silica gel 60 $\text{NH}_2 \text{ F}_{254\text{S}}$ (art. no. 1.13192, Merck) plates were twofold pre-developed. Silica gel and NH_2 plates were firstly pre-developed with methanol–formic acid in the ratio of 10:3 (V/V) and 10:5 (V/V), respectively, and dried in a stream of warm air (a hair dryer) for 5 min. After drying, all plates were secondly pre-developed with methanol. After the second pre-development, the plates were dried in an oven at 110°C for 30 min and cut to $10 \text{ cm} \times 10 \text{ cm}$ pieces that were used for HPTLC– MS^n analyses. STSs (STS–SI and STS–TR; $150 \mu\text{L}$, 100 mg/mL) were applied by means of a Linomat 5 (CAMAG, Muttenz, Switzerland) on separate plates as a 60 mm band, 8 mm from the bottom of the plate. Silica gel and NH_2 plates were developed in 20 min up to 7 cm in a saturated (15 min) twin-trough chamber with ethyl

acetate–formic acid–acetic acid–water (10:1.1:1.1:2.6, V/V) [26] and ethyl acetate–dichloromethane–formic acid–acetic acid (10:2.5:1:1.1, V/V), respectively. After development and drying in a stream of warm air (2 min), the left-most part (2 cm) of the plates was heated at 110 °C [on a TLC plate heater 3 (CAMAG)] for 2 min, and immersed into a natural product (NP) detection reagent containing diphenylboric acid 2-aminoethyl ester (1 g) in ethyl acetate (200 mL) [27]. After drying in a stream of warm air for 2 min and cooling, the plates were immersed into PEG detection reagent prepared by dissolving PEG 4000 (10 g) in dichloromethane (200 mL) [28] and dried in a stream of warm air for 2 min. Chromatograms were documented by means of the DigiStore 2 documentation system (CAMAG) at 366 nm and white light after development and post-chromatographic derivatisation with NP reagent and after enhancement and stabilisation of fluorescent zones with PEG reagent.

The derivatised part of the plate enabled the appropriate positioning of the desired chromatographic zones under the elution head (4 mm × 2 mm) of the TLC–MS interface (CAMAG), that was used for elution of analytes from the plates into LTQ Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). An in-line filter 0.5 µm (Idex, Health & Science, Oak Harbor, WA, USA) was mounted between the TLC–MS interface and ion source of the mass spectrometer to ensure additional protection of the mass spectrometer against particles of the stationary phase. The flow rate of methanol, that was used as the eluent, was 0.2 mL/min. Heated electrospray ionisation (HESI) in the negative ion mode was used for ionisation of the compounds. The MS parameters were as follows: heater and capillary temperature 200 °C and 350 °C, respectively; sheath gas 60 arbitrary units (a.u.); auxiliary gas 10 a.u.; sweep gas 0 a.u.;

spray voltage 2.5 kV; capillary voltage 38.8 V and S-Lens RF level 69.0% [18]. Target precursor ions were fragmented in MSⁿ experiments using a collision energy of 40%. MS spectra were acquired in the *m/z* range of 100–2000. Xcalibur software (Version 2.1.0, Thermo) was applied to evaluate the collected data.

3 Results and discussion

In this study, HPTLC silica gel plates were developed with the developing solvent ethyl acetate–formic acid–acetic acid–water (10:1.1:1.1:2.6, V/V). This developing solvent contained formic and acetic acid, which caused intense background signals of sodium formate clusters (with $\Delta m/z$ 68) and sodium acetate clusters (with $\Delta m/z$ 82) in the MS spectrum (Fig. 1a). The same problem was observed on NH₂ plates (modified silica gel) developed with ethyl acetate–dichloromethane–formic acid–acetic acid (10:2.5:1:1.1, V/V), which also contained formic and acetic acid that caused intense background signals of sodium formate clusters (with $\Delta m/z$ 68) (Fig. 2a). Based on previous HPTLC–MS and HPTLC–MSⁿ studies [14, 16–23], in which the developing solvent mixtures contained only formic acid, it was predicted that methanol–formic acid (10:1, V/V, in the first pre-development step) would not be sufficient to eliminate sodium formate and sodium acetate clusters. Therefore, the following ratios of methanol–formic acid: 10:1.5, 10:3, 10:5 (V/V) were tested on silica gel and NH₂ plates. It was observed that increasing the concentration of formic acid in the pre-developing solvent resulted in improved background with less pronounced ions of acid clusters. Acid clusters were successfully removed from the layer of silica

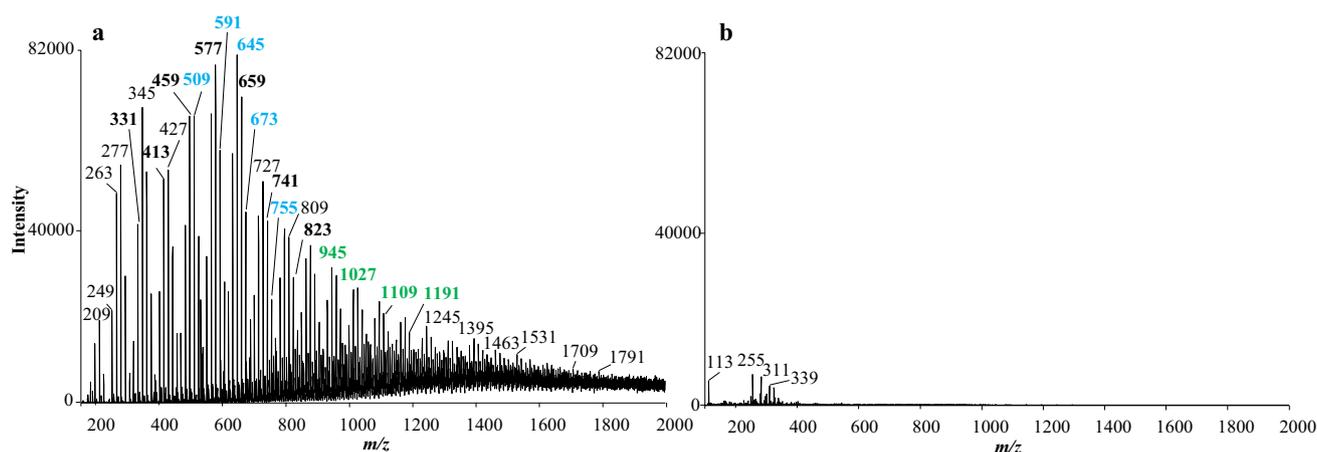


Fig. 1 MS spectra of backgrounds obtained at $R_F=0.71$ from not pre-developed (**a**) and twofold pre-developed (**b**; first: methanol–formic acid (10:3, V/V); second: methanol) HPTLC silica gel plates developed with ethyl acetate–formic acid–acetic acid–water

(10:1.1:1.1:2.6, V/V). Sets of background signals of sodium acetate clusters with $\Delta m/z$ 82 are in bold, with *m/z* numbers in black, blue and green

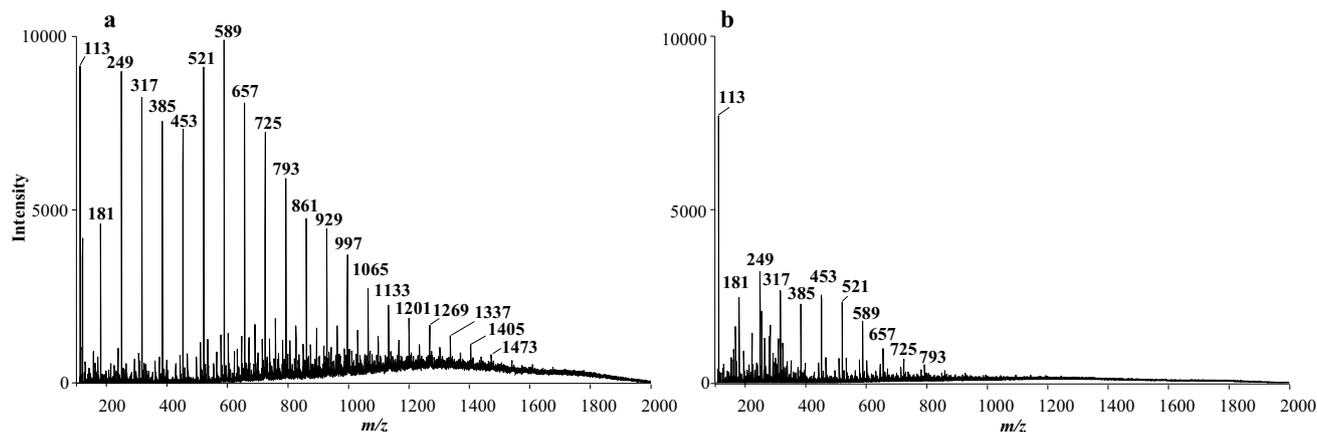


Fig. 2 MS spectra of backgrounds obtained at $R_F=0.71$ from not pre-developed (a) and twofold pre-developed (b; first: methanol–formic acid (10:5, V/V); second: methanol) HPTLC NH_2 plates devel-

oped with ethyl acetate–dichloromethane–formic acid–acetic acid (10:2.5:1:1.1, V/V). Sets of background signals of sodium formate clusters with $\Delta m/z$ 68

gel plates by pre-development with methanol–formic acid 10:3 (V/V) (Fig. 1b). In the case of NH_2 plates, acid clusters were observed in the MS spectrum even after pre-development with methanol–formic acid 10:5 (V/V) (Fig. 2b), but their intensity had dropped significantly. Based on previous studies [14, 16–23], the second pre-development of silica gel and NH_2 plates was performed with methanol that was intended for use as an elution solvent in HPTLC– MS^n experiments. The developed pre-development procedures enabled HPTLC– MS^n analyses of chestnut bee pollen samples on silica gel and NH_2 plates without any problem related to ion suppression of the desired analytes.

Possible influence of the pre-developing solvents on the separation on silica gel plates and NH_2 plates was examined before HPTLC– MS^n analyses of STSs. This examination was performed by HPTLC analyses of STS–SI on silica gel (Fig. 3a–d) and NH_2 (Fig. 3e–h) plates that were not pre-developed (Fig. 3a, b, e, f) or were twofold pre-developed (Fig. 3c, d, g, h). Silica gel plates were pre-developed with methanol–formic acid (10:3, V/V) and methanol, while NH_2 plates were pre-developed with methanol–formic acid (10:5, V/V) and methanol. Silica gel plates were developed with ethyl acetate–formic acid–acetic acid–water (10:1.1:1.1:2.6, V/V) (Fig. 3a–d) and NH_2 plates

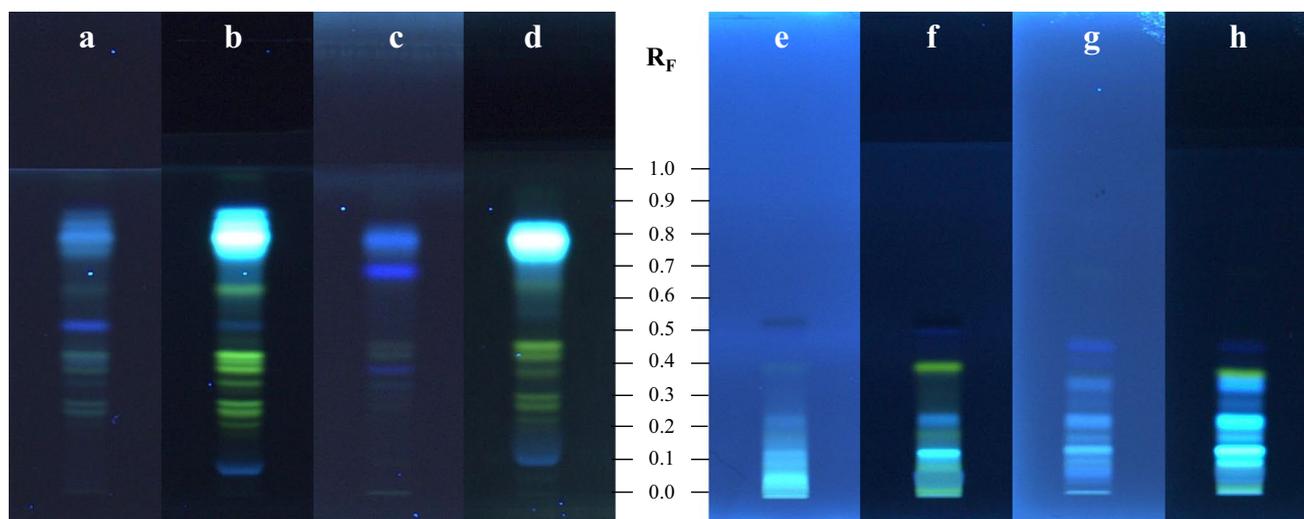


Fig. 3 Chromatograms of STS–SI (100 mg/mL, 1 μL) on not pre-developed (a, b, e, f) and twofold pre-developed (c, d, g, h) HPTLC silica gel (a–d) and NH_2 (e–h) plates developed with ethyl acetate–formic acid–acetic acid–water (10:1.1:1.1:2.6, V/V) (a–d) and ethyl

acetate–dichloromethane–formic acid–acetic acid (10:2.5:1:1.1, V/V) (e–h), respectively. Documentation was performed at 366 nm after derivatisation with NP reagent and after enhancement and stabilisation of fluorescent zones with PEG reagent

with ethyl acetate–dichloromethane–formic acid–acetic acid (10:2.5:1:1.1, V/V) (Fig. 3e–h). Both plates were derivatised with NP reagent while enhancement and stabilisation of the fluorescent chromatographic zones were achieved with PEG reagent. Pre-development influenced the separation resulting in (1) a different number of the separated chromatographic zones and (2) different resolutions (Fig. 3). The effect of plate pre-development was more pronounced on NH_2 than on silica gel plates. A lower number of blue fluorescent and green fluorescent zones were observed on twofold pre-developed (Fig. 3c, d) than on not pre-developed (Fig. 3a, b) HPTLC silica gel plates. A lower number of green fluorescent zones and a higher number of blue fluorescent zones were observed on twofold pre-developed (Fig. 3g, h) than on not pre-developed (Fig. 3e, f) HPTLC NH_2 plates. Changes between R_F values for particular chromatographic zones on not pre-developed (Fig. 3e, f) and on twofold pre-developed (Fig. 3g, h) NH_2 plates were much larger than changes between R_F values on not pre-developed (Fig. 3a, b) and on twofold pre-developed (Fig. 3c, d) silica gel plates.

Non-targeted HPTLC–MSⁿ analyses of chestnut bee pollen test solutions from Slovenia (STS–SI) and Türkiye (STS–TR) were performed on twofold pre-developed silica gel plates. Compounds were tentatively identified by comparing fragmentation patterns of MS² (Fig. 4, Table 1), MS³, MS⁴ and MS⁵ (Table 1) spectra with those published in the literature. The molecular ion at m/z 582 $[\text{M}–\text{H}]^-$ with MS² fragments at m/z 462, m/z 342 and m/z 436 with neutral losses of 120 u, 240 u and 146 u, respectively, which corresponded to coumaroyl moiety at positions N^1 , N^5 and N^{10} [29], was assigned as N^1, N^5, N^{10} -tricomaroylspermidine (Fig. 5) based on the literature [11, 13, 29, 30]. The molecular ion at m/z 598 was previously assigned as $[\text{M}–\text{H}]^-$ of N^1, N^5 -dicoumaroyl- N^{10} -caffeoylspermidine in the extracts of chestnut bee pollen [11] and chestnut bee bread [30] or as N^1, N^{10} -dicoumaroyl- N^5 -caffeoylspermidine in the extracts of chestnut bee pollen [11]. Fragmentation of the molecular ion at m/z 598 can yield base ions at 478 m/z [11] or at 462 m/z [11, 30]. In this study only a base ion at 478 m/z was detected. Similarly, as in other studies [11, 30], a base ion at 478 m/z was also observed in MS² experiments after fragmentation of spermidines bonding caffeoyl moiety at position N^5 . Three other spermidine derivatives with caffeoyl moiety at position N^5 also had similar fragmentation patterns in MS⁴ and MS⁵ experiments as the molecular ion at 598 m/z . Therefore, the molecular ion at 598 m/z was assigned as $[\text{M}–\text{H}]^-$ of N^1, N^{10} -dicoumaroyl- N^5 -caffeoylspermidine [11, 13] (Fig. 5).

Fragmentation of the molecular ion at m/z 614 $[\text{M}–\text{H}]^-$ resulted in the ions at m/z 478, 452 and 494, with neutral losses of 136 u, 162 u and 120 u, respectively. Fragmentation ions at m/z 478 and 452 corresponded to caffeoyl residue at position N^{10} and N^5 , respectively [29],

while fragmentation ions at m/z 494 corresponded to *p*-coumaroyl residue at position N^1 . Therefore, the molecular ion at m/z 614 $[\text{M}–\text{H}]^-$ was assigned as N^1 -coumaroyl- N^5, N^{10} -dicaffeoylspermidine (Fig. 5) based on the literature [11, 13, 30]. The molecular ion at m/z 644 $[\text{M}–\text{H}]^-$ fragmented in ions at m/z 508 and 482 with neutral losses of 136 u and 162 u, respectively, which corresponded to caffeoyl residue at position N^{10} and N^5 , respectively [29].

An additional MS² ion at m/z 494 with neutral loss of 150 u suggested the feruloyl residue at position N^1 [29]. Therefore, the molecular ion at m/z 644 $[\text{M}–\text{H}]^-$ was assigned as $[\text{M}–\text{H}]^-$ of N^1 -feruloyl- N^5, N^{10} -dicaffeoylspermidine [11, 30, 31] (Fig. 5). The molecular ion at m/z 630 $[\text{M}–\text{H}]^-$ with MS² fragments at m/z 468, m/z 494 and m/z 358 with neutral losses of 162 u, 136 u and 272 u, respectively, which corresponded to caffeoyl moiety at positions N^1 , N^5 and N^{10} [29], was assigned as N^1, N^5, N^{10} -tricomaroylspermidine [11, 29, 30] (Fig. 5).

Out of the group of flavonols, only glycosides of isorhamnetin (with signals at m/z 314/315 $[\text{M}–2\text{H}]^-$) [12] were identified (Fig. 4, Table 1). The molecular ion at m/z 477 with neutral loss of 162 u, which corresponded to hexose residue, was assigned as $[\text{M}–\text{H}]^-$ of isorhamnetin hexoside [11, 30]. Fragmentation of the molecular ion at m/z 519 resulted in signals at m/z 459, 315 and 477. Neutral loss of 204 u of the molecular ion at m/z 519 corresponded to hexosyl and acetyl moieties, while the signal at m/z 477 corresponded to isorhamnetin hexoside. Therefore, the signal at m/z 519 was assigned as $[\text{M}–\text{H}]^-$ of isorhamnetin acetylhexoside [32]. The molecular ion at m/z 639 $[\text{M}–\text{H}]^-$ [33] with neutral loss of 324 u corresponded to dimer of hexoses, and was tentatively identified as isorhamnetin-(hexosyl)hexoside. The molecular ion at m/z 609 with neutral loss of 294 u, which corresponded to pentosylhexosyl moiety, was assigned as $[\text{M}–\text{H}]^-$ of isorhamnetin-(pentosyl)hexoside [11, 30, 34]. The molecular ion at m/z 623 $[\text{M}–\text{H}]^-$ fragmented into ions at m/z 314/315 (corresponding to isorhamnetin) with neutral loss of 308 u, which corresponded to hexosylpentosyl [12] or coumaroylhexosyl moiety [35]. Additional ions obtained in MS² spectra were also at m/z 459 and 477, which corresponded to the neutral loss of hexosyl and deoxyhexosyl residues, respectively. Therefore, the molecular ion at m/z 623 was assigned as $[\text{M}–\text{H}]^-$ of isorhamnetin-(deoxyhexosyl)hexoside [11, 30]. The molecular ion at m/z 755 $[\text{M}–\text{H}]^-$ fragmented into the ions at m/z 314/315 (corresponding to isorhamnetin) with neutral loss of 440 u. Two characteristic ions at m/z 609 and 623 in the MS² spectrum corresponded to isorhamnetin-(pentosyl)-hexoside and isorhamnetin-(deoxyhexosyl)hexoside; therefore, the molecular ion at m/z 755 $[\text{M}–\text{H}]^-$ can be isorhamnetin-(pentosyl)-deoxyhexosyl)hexoside.

After the MS² fragmentation, the molecular ion at m/z 377 $[\text{M}–\text{H}]^-$ lost two molecules of water. The base ion

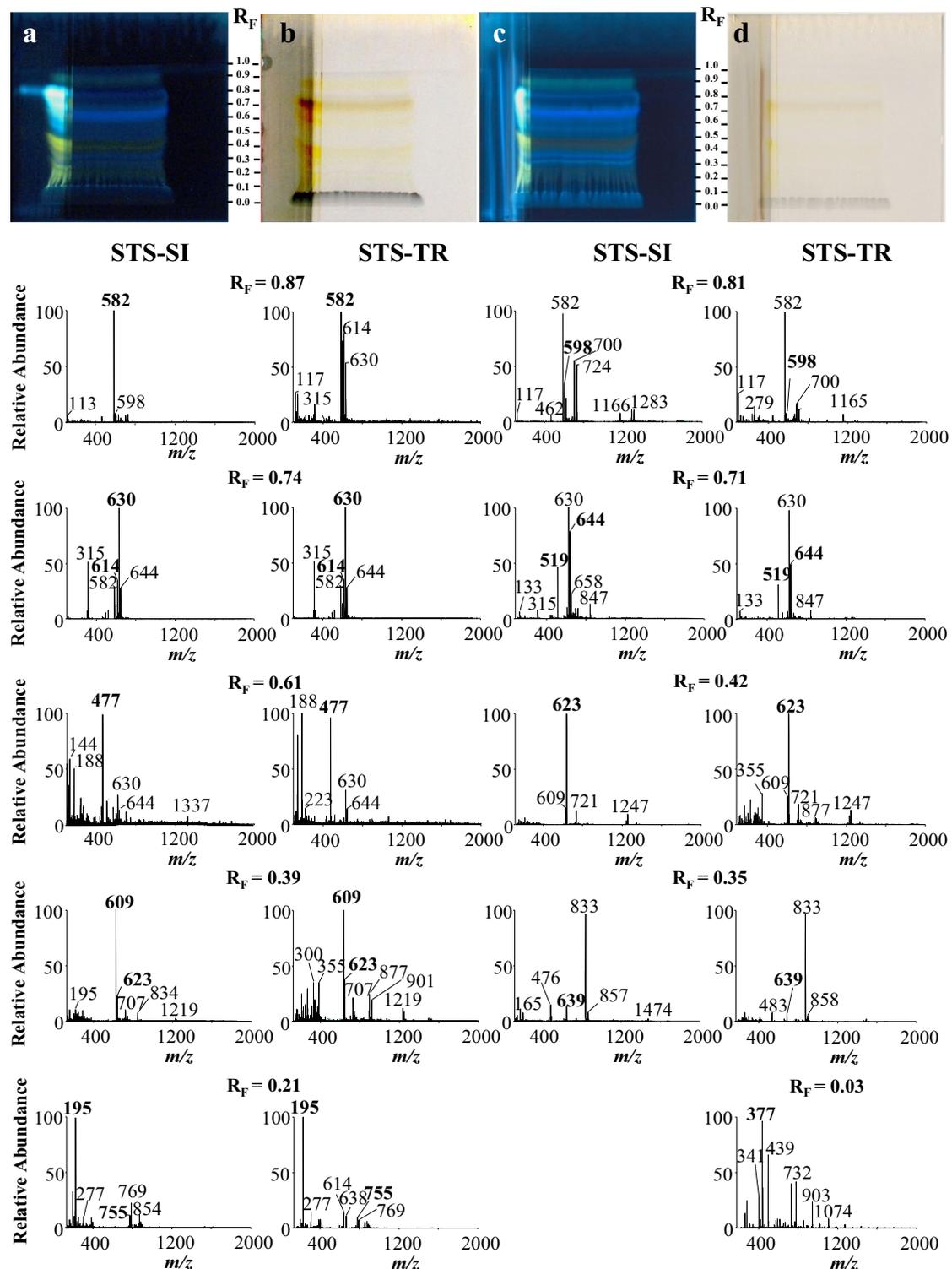


Fig. 4 HPTLC–MS analyses of STS–SI (**a**, **b**) and STS–TR (**c**, **d**) on twofold pre-developed [first methanol–formic acid, 10:3 (V/V), second methanol] HPTLC silica gel plates developed with ethyl acetate–formic acid–acetic acid–water (10:1.1:1.1:2.6, V/V) and documented at 366 nm (**a**, **c**) and white light (**b**, **d**) after derivatisation of the left-most part with NP and PEG reagents. MS spectra with bolded signals of $[M-H]^-$: N^1,N^5,N^{10} -tricoumaroylspermidine (m/z 582), N^1,N^{10} -dicoumaroyl- N^5 -caffeoylspermidine (m/z 598),

$[M-H]^-$ at m/z 630), N^1 -coumaroyl- N^5,N^{10} -dicafeoylspermidine (m/z 614), N^1 -feruloyl- N^5,N^{10} -dicafeoylspermidine (m/z 644), isorhamnetin-acetylhexoside (m/z 519), isorhamnetin-hexoside (m/z 477), isorhamnetin-(pentosyl)hexoside (m/z 609), isorhamnetin-(hexosyl)deoxyhexoside (m/z 623), isorhamnetin-(hexosyl)hexoside (m/z 639) gluconic acid (m/z 195), isorhamnetin-(pentosyl-deoxyhexosyl)hexoside (m/z 755), caffeic acid-hexoside ($[M-2H_2O]^-$ at m/z 377)

Table 1 Phenylamides, flavonols, phenolic and organic acids tentatively identified by HPTLC–ESI/MSⁿ analyses of chesnut bee pollen (STS–SI and STS–TR) on pre-developed HPTLC silica gel and NH₂ plates

Compound	[M–H] [–] (<i>m/z</i>)	MS ⁿ fragmentation (<i>m/z</i>)	NH ₂		Silica gel			References
			<i>R</i> _F	STS– SI	STS– SI	STS– TR	<i>R</i> _F	
Phenylamides								
<i>N</i> ¹ , <i>N</i> ⁵ , <i>N</i> ¹⁰ -tricoumaroyl-spermidine	582	MS ² [582]: 462, 342, 436, 316, 279, 419 MS ³ [582 → 462]: 342, 419, 316, 145 MS ⁴ [582 → 462 → 342]: 299, 145	0.52	+	+	+	0.87	[11, 13, 30]
<i>N</i> ¹ , <i>N</i> ¹⁰ -dicoumaroyl- <i>N</i> ⁵ -caffeoylspermidine	598	MS ² [598]: 478, 462, 452, 342, 358, 436, 315, 332 MS ³ [598 → 478]: 358, 342, 332, 435, 315 MS ⁴ [598 → 478 → 358]: 315, 161, 272 MS ⁵ [598 → 478 → 358 → 315]: 272, 160, 175, 218, 161, 273, 159, 287, 228, 135, 297	0.52	+	+	+	0.81	[11, 13]
<i>N</i> ¹ , <i>N</i> ⁵ , <i>N</i> ¹⁰ -tricafeoyl-spermidine	630	MS ² [630]: 468, 494, 358, 332 MS ³ [630 → 468]: 332, 306 MS ⁴ [630 → 468 → 332]: 289, 135 MS ⁵ [630 → 468 → 332 → 289]: 247, 135, 261, 235, 246, 177, 204, 289 MS ³ [630 → 494]: 358, 332 MS ⁴ [630 → 494 → 358]: 315, 161, 272 MS ⁵ [630 → 494 → 358 → 315]: 272, 160, 271, 175	0.32	+	+	+	0.74	[11, 13, 30]
<i>N</i> ¹ -coumaroyl- <i>N</i> ⁵ , <i>N</i> ¹⁰ -dicaffeoylspermidine	614	MS ² [614]: 478, 452, 358, 494, 468, 332, 316, 342 MS ³ [614 → 478]: 358, 342, 332 MS ⁴ [614 → 478 → 358]: 315, 161, 272 MS ⁵ [614 → 478 → 358 → 315]: 272, 175, 161, 160, 271, 218, 273	0.42	+	+	+	0.74	[11, 30, 31]
<i>N</i> ¹ -feruloyl- <i>N</i> ⁵ , <i>N</i> ¹⁰ -dicaffeoylspermidine	644	MS ² [644]: 508, 482, 468, 358, 332, 494, 372, 346 MS ³ [644 → 508]: 358, 372, 332, 465 MS ⁴ [644 → 508 → 358]: 315, 161 MS ⁵ [644 → 508 → 358 → 315]: 272, 159, 175	0.42	+	+	+	0.71	[11, 30, 31]
Flavonols								
Isorhamnetin-hexoside	477	MS ² [477]: 314, 315, 357, 285, 271, 299 MS ³ [477 → 314]: 285, 271, 286, 243, 257, 299 MS ⁴ [477 → 314 → 285]: 270	0.32	+	+	+	0.61	[11, 30, 32, 37]
Isorhamnetin-acetylhexoside	519	MS ² [519]: 459, 315, 299, 477 MS ³ [519 → 459]: 315, 314, 299, 313, 285 MS ⁴ [519 → 459 → 315]: 300 MS ⁵ [519 → 459 → 315 → 300]: 271, 272, 255, 151, 256, 227	0.52	+	+	+	0.71	[32, 39]
Isorhamnetin-(pentosyl)-hexoside	609	MS ² [609]: 315, 300, 314, 540, 459, 271, 299, 472 MS ³ [609 → 315]: 300, 287, 272, 256 MS ⁴ [609 → 315 → 300]: 271, 255, 272, 254, 243, 151	0.06	+	+	+	0.39	[11, 30, 34, 37]
Isorhamnetin-(hexosyl)-deoxy-hexoside	623	MS ² [623]: 314, 315, 459, 299, 300, 271, 357, 591, 477 MS ³ [623 → 314]: 299, 271, 285, 286, 243 MS ⁴ [623 → 314 → 299]: 271, 255, 243, 227, 199 MS ⁵ [623 → 314 → 299 → 279]: 243, 198, 227, 214, 271	0.06	+	+	+	0.42	[11, 12, 30, 34]
Isorhamnetin-(hexosyl)-hexoside	639	MS ² [639]: 315, 314, 459, 300, 299, 271, 477, 519, 607 MS ³ [639 → 315]: 300, 287, 151	0.06	+	+	+	0.35	[12, 33]

Table 1 (continued)

Compound	[M–H] [–] (<i>m/z</i>)	MS ⁿ fragmentation (<i>m/z</i>)	NH ₂		Silica gel		References
			R _F	STS–SI	STS–SI	STS–TR	
Isorhamnetin-(pentosyl)-deoxy-hexosyl)-hexoside	755	MS ² [755]: 314, 315, 591, 299, 357, 271, 503, 399, 423, 609, 623 MS ³ [755 → 314]: 299, 271, 286	0.06	+	+	+	0.21 /
Phenolic acids							
Caffeic acid-hexoside [M–2H ₂ O] [–]	377	MS ² [377]: 341, 215, 179 MS ³ [377 → 341]: 179, 161, 143, 112, 119, 101, 281, 149	/	–	–	+	0.03 [36]
Organic acids							
Gluconic acid	195	MS ² [195]: 129, 177, 159, 99, 75, 87 MS ³ [195 → 129]: 85, 57, 101 MS ⁴ [195 → 129 → 85]: 57, 178, 140	0.06	+	+	+	0.21 [16, 40]

+detected

– not detected

obtained at *m/z* 341 further fragmented with a neutral loss of 162 u (corresponding to hexosyl moiety) [36] into a base ion at *m/z* 179, characteristic for caffeic acid [37]. Therefore, the molecular ion at *m/z* 377 was tentatively assigned as [M–2H₂O][–] of caffeic acid-hexoside. The fragmentation pattern of the molecular ion at *m/z* 195 [M–H][–] was similar as previously reported in honey [38] and rose hip [16]; therefore, the signal at *m/z* 195 was tentatively assigned as [M–H][–] of gluconic acid.

The applicability of the pre-developed NH₂ plates for on-line HPTLC–MSⁿ analyses of bee pollen samples was examined by the analyses of STS–SI. It was observed that the same phenylamides and glycosylated flavonols were identified in STS–SI using NH₂ and silica gel plates. Differences between NH₂ and silica gel plates were found in some of the unidentified compounds (Table 2). Some of those compounds (at *m/z* 114, 292, 315) were only detected when using NH₂ plates, and other compounds (at *m/z* 165, 188, 279, 355, 439, 461, 745, 833) only when using silica gel plates. Comparing both types of the plates, more interferences were observed between compounds detected on NH₂ plates (Tables 1, 2).

Five phenylamides, six glycosylated flavonols and one organic acid were identified in the STSs of both chestnut bee pollen samples (Fig. 4, Table 1). One glycosylated phenolic acid was detected only in the STS–TR (Fig. 4, Table 1). Isorhamnetin hexoside, isorhamnetin-(pentosyl) hexoside, isorhamnetin-(hexosyl)deoxyhexoside and all phenolspermidines identified in this study (Fig. 4, Table 1) were recently also found in chestnut bee pollen samples [11]. Isorhamnetin-(hexosyl)hexoside (Fig. 4, Table 1) was previously identified in multi-floral bee pollen samples [12] and in honey collected by sugarbag bee (*Tetragonula carbonaria* Smith) [33]. Isorhamnetin-acetylhexoside (Fig. 4, Table 1) was previously identified in chestnut flowers [39].

To the best of our knowledge, this is the first report on isorhamnetin-(hexosyl)hexoside, isorhamnetin-acetylhexoside, isorhamnetin-(pentosyl-deoxyhexosyl)hexoside and caffeic acid-hexoside in chestnut bee pollen. This is also the first report on isorhamnetin-(pentosyl-deoxyhexosyl) hexoside and caffeic acid-hexoside in any bee products.

Fig. 5 The chemical structures of phenylamides identified in chestnut bee pollen samples

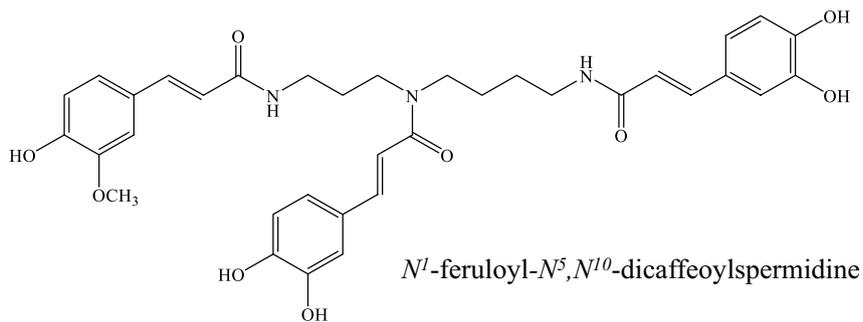
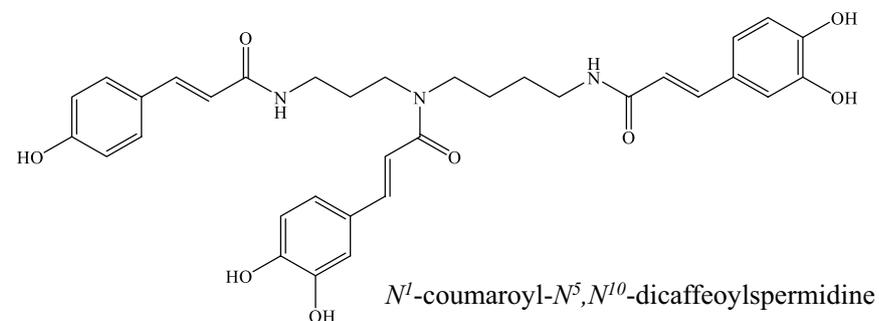
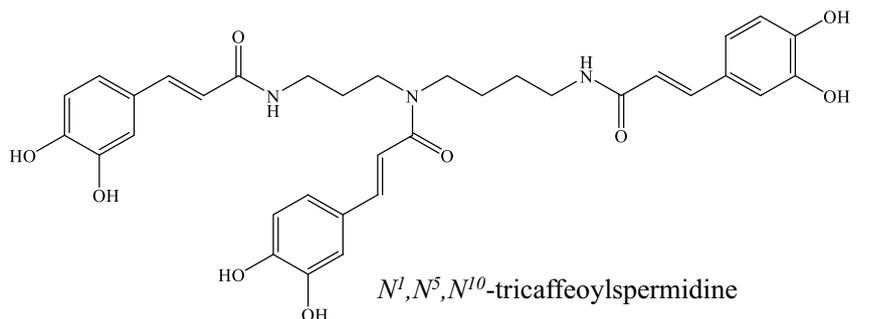
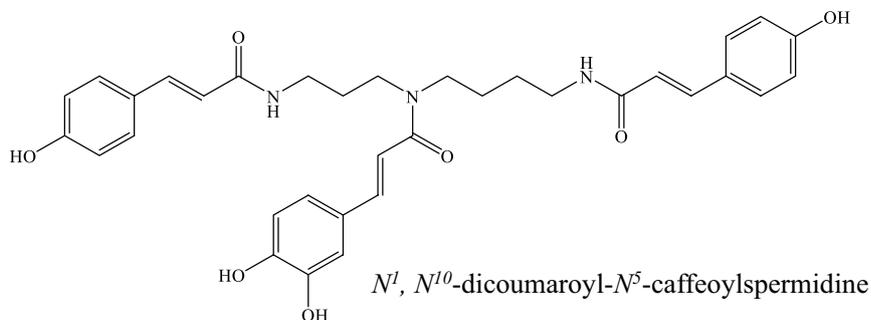
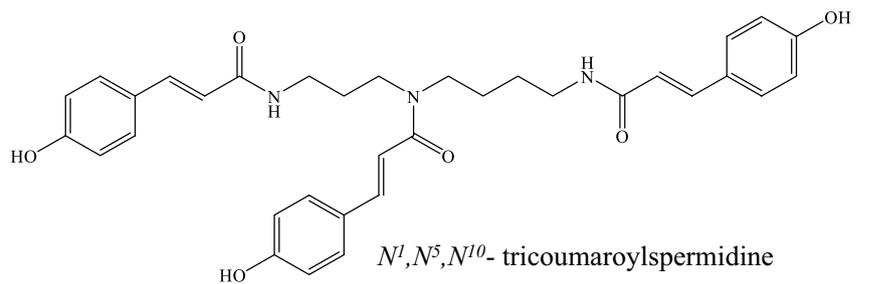


Table 2 Unknown compounds obtained by HPTLC–ESI/MSⁿ analyses of chestnut bee pollen (STS–SI and STS–TR) on pre-developed HPTLC silica gel and NH₂ plates

[M–H] [–] (<i>m/z</i>)	MS ⁿ fragmentation (<i>m/z</i>)	NH ₂		Silica gel		
		<i>R</i> _F	STS–SI	STS–SI	STS–TR	<i>R</i> _F
114	MS ² [114]: 68, 86, 96, 85	0.23	+	–	–	/
165	MS ² [165]: 147, 129, 75, 99, 85, 101, 57, 119 MS ³ [165 → 147]: 129, 99, 118, 57, 117, 101, 85 MS ⁴ [165 → 147 → 129]: 85, 57, 101, 73, 111, 69	/	–	+	+	0.23
188	MS ² [188]: 144 MS ³ [188 → 144]: 102, 126, 116, 160, 66, 115, 131	/	–	+	+	0.65
223	MS ² [223]: 195, 179, 177, 205, 196, 208, 181, 194, 129, 193	0.34	+	+	+	0.57
279	MS ² [279]: 261, 259, 243, 235 MS ³ [279 → 261]: 243, 259, 219, 233, 83, 205, 97, 165, 109	/	–	+	+	0.94
292	MS ² [292]: 177, 129, 114, 159, 202, 248, 264	0.23	+	–	–	/
294	MS ² [294]: 174, 186, 226, 204, 276, 250, 157, 228 MS ³ [294 → 174]: 144, 156, 146, 268, 79	0.19	+	+	+	0.13
315	MS ² [315]: 135, 247, 161, 234, 136, 300	0.32	+	–	–	/
355	MS ² [355]: 309, 311, 273, 337 MS ³ [355 → 309]: 305, 281, 307, 287	/	–	+	+	0.52
439	MS ² [439]: 421, 341, 277, 393, 355, 259, 359, 365, 411, 367, 195	/	–	–	+	0.10
461	MS ² [461]: 279 MS ³ [461 → 279]: 261	/	–	+	–	0.55
699	MS ² [699]: 279, 277, 255, 278, 437, 438, 256, 419	0.23	+	+	+	0.86
715	MS ² [715]: 279, 255, 452, 280, 256, 453, 476, 434 MS ³ [715 → 279]: 261, 243, 262, 259	0.35	+	–	+	0.54
745	MS ² [745]: 279, 255, 277, 278, 256, 391, 483, 465, 488 MS ³ [745 → 279]: 261, 243	/	–	+	+	0.52
749	MS ² [749]: 739, 737, 738, 736, 731, 666, 734, 438 MS ³ [749 → 739]: 716	0.52	+	+	+	0.66
826	MS ² [826]: 766, 765, 367, 279	0.48	+	+	–	0.26
833	MS ² [833]: 553, 554, 391, 255, 279, 577, 571, 297, 241, 415, 554, 409 MS ³ [833 → 553]: 223, 255, 391, 297, 241, 497, 259, 392, 461, 315 MS ⁴ [833 → 553 → 223]: 79, 97, 205	/	–	+	+	0.32

+ detected

– not detected

4 Conclusions

Two types of the HPTLC plates (silica gel and NH₂ stationary phases) in combination with developing solvents containing formic and acetic acid (both at the same time) were examined for HPTLC–MSⁿ analyses of chestnut bee pollen samples from Slovenia and Türkiye. This paper demonstrates that, due to acid clusters, these plates cannot be used for such analyses without pre-development. The developed HPTLC–MSⁿ methods are based on pre-developing procedures that include two steps. In the first step, pre-development of the silica gel plates is performed with methanol–formic acid (10:3, V/V), and pre-development of NH₂ plates with methanol–formic acid (10:5, V/V). Then the second pre-development step with methanol is equal for both types of the plates. This twofold pre-development of

the plates significantly improves the background by removing the acid clusters (silica gel plates) or at least significantly decreasing the intensity of those clusters (NH₂ plates) thus enabling HPTLC–MSⁿ analyses of chestnut bee pollen analyses on both types of the plates. Using the developed HPTLC–MSⁿ methods, five phenylamides, six isorhamnetin glycosides and one organic acid (gluconic acid) were identified in chestnut bee pollen samples from Slovenia and Türkiye. Glycosylated phenolic acid (caffeic acid-hexoside) was detected only in the Turkish bee pollen sample. To the best of our knowledge, this is the first report on isorhamnetin-(hexosyl)hexoside, isorhamnetin-acetylhexoside, isorhamnetin-(pentosyl-deoxyhexosyl)hexoside and caffeic acid-hexoside in chestnut bee pollen. This is also the first report on isorhamnetin-(pentosyl-deoxyhexosyl)hexoside and caffeic acid-hexoside in any bee products.

The developed analytical methodology offers a variety of possible applications such as: (1) discovery of analysed compounds in other bee pollens and flower pollen samples, (2) the discovery of biomarkers characteristic for specific bee pollens, (3) the development of new food supplements and other products and (4) characterisation of bee pollen which can be applied also in clinical trials investigating the potential health benefits of bee pollen.

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Author contributions Conceptualisation: IV, VG, EG; Methodology: IV, VG; Formal analysis and investigation: VG, MB, IV; Writing – original draft preparation: VG, IV, MB; Writing – review and editing: VG, IV, EG, MB; Funding acquisition: IV, EG; Resources: IV, EG.

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Data availability On request.

Code availability Not applicable.

Declarations

Conflict of interest The first author, Vesna Glavnik and the third author, Irena Vovk are members of the Editorial Board of the journal. Therefore, the submission was handled by a different member of the editorial board, and they did not take part in the review process in any capacity.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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